Molecular and physiological regulation of lifespan, ageing and division of labour in social insects

Dissertation

Zur Erlangung des Grades Doktor der Naturwissenschaften

Am Fachbereich Biologie

Der Johannes Gutenberg-Universität Mainz

Vorgelegt von

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geb. am 15.05.1994 in Burgwedel

Mainz, 2026

Dekan: [Removed for privacy purposes]

1. Berichterstatterin: [Removed for privacy purposes]

2. Berichterstatter: [Removed for privacy purposes]

Tag der mündlichen Prüfung: [Removed for privacy purposes]

“To live would be an awfully big adventure.”

— Peter Pan

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Zusammenfassung

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Im Zentrum dieser Dissertation steht die Frage, inwiefern Reproduktion und soziale Umwelt die Lebensdauer von Ameisen beeinflussen und welche Bedeutung dabei das reproduktive Potenzial der Arbeiterinnen hat. Hierzu wurden vier Ameisenarten mit stark divergierenden Sozial- und Fortpflanzungssystemen untersucht. Mithilfe experimenteller Manipulationen der Königinnenanzahl und der Reproduktionsaktivität sowie durch vergleichende Analysen von Überleben, Ovarentwicklung, oxidativer Stressresistenz und Genexpression konnten kausale Zusammenhänge zwischen Reproduktion, Physiologie und Lebensdauer aufgezeigt werden. In **Kapitel 1** wird am Beispiel der hochpolygynen, invasiven Ameise *Tapinoma magnum* untersucht, wie die Königinnenanzahl das Überleben, die Reproduktion und die Physiologie von Arbeiterinnen beeinflusst. Arbeiterinnen lebten am längsten in Kolonien mit einer Königin, während königinnenlose Arbeiterinnen die höchste Mortalität aufwiesen. Unterschiede in oxidativer Stressresistenz und Genexpression waren jedoch stärker mit der Arbeitsteilung (Nest- versus Außenarbeiterinnen) als mit der Königinnenanzahl verknüpft. Königinnen zeigten über verschiedene Altersstufen hinweg eine ausgeprägte physiologische Stabilität. In **Kapitel 2** wird die klonale Räuberameise *Ooceraea biroi* genutzt, um den kausalen Zusammenhang zwischen Reproduktion und Lebensdauer experimentell zu testen. Durch gezielte Unterdrückung der Reproduktion konnte gezeigt werden, dass nicht-reproduktive Arbeiterinnen eine verkürzte Lebensdauer aufweisen, während kontinuierliche oder zyklische Reproduktion mit erhöhter Überlebenswahrscheinlichkeit einhergeht. Reproduktive Arbeiterinnen zeigten zudem eine verstärkte Expression von Genen, die mit antioxidativem Schutz, Immunfunktion und DNA-Reparatur assoziiert sind, was einen direkten Nachweis für die Umkehr des klassischen „Longevity/Fecundity“-Trade-offs liefert. **Kapitel 3** untersucht die Auswirkungen des Königinnenverlusts in der Ameise *Messor capitatus*, einer Art mit potenziell totipotenten Arbeiterinnen. Obwohl der Königinnenverlust zu einer Aktivierung der Ovarien führte, resultierte dies nicht konsistent in einer erhöhten Lebensdauer. Stattdessen erwies sich die Koloniegröße als entscheidender Faktor für das Überleben, was auf kontextabhängige Grenzen lebensverlängernder Effekte von Reproduktion hinweist. In **Kapitel 4** wird mit *Hypoponera opacior* eine Art mit obligat sterilen Arbeiterinnen untersucht. Hier zeigte sich kein Effekt der Königinnenabwesenheit auf die Lebensdauer der Arbeiterinnen, was darauf hindeutet, dass reproduktives Potenzial eine

zentrale Voraussetzung für lebensverlängernde Effekte nach Königinnenverlust ist. **Kapitel 5** befasst sich mit der genetischen Grundlage alternativer reproduktiver Strategien in *H. opacior*. Genomische Analysen lieferten keine Hinweise auf eine supergenbasierte Fixierung, sondern sprechen für eine plastische, umweltabhängige Regulation der Reproduktionsmorphologie.

Zusammenfassend zeigt diese Dissertation, dass Altern und Lebensdauer in Ameisen maßgeblich durch das Zusammenspiel von sozialer Organisation, reproduktivem Status und physiologischer Plastizität bestimmt werden. Die Ergebnisse verdeutlichen, dass eine Auflösung des klassischen Trade-offs zwischen Reproduktion und Langlebigkeit möglich ist, wenn Reproduktion in einen sozial geschützten Kontext eingebettet ist, und liefern neue Einblicke in die evolutionären und mechanistischen Grundlagen des Alterns.

Summary

Summary

This dissertation investigates how ageing and lifespan in ants are shaped by social organisation, reproductive status, and physiological plasticity. **Chapter 1** examines how queen number affects worker survival, reproduction, and physiology in the highly polygynous, invasive ant *Tapinoma magnum*. Worker survival was highest in colonies with a single queen and lowest in queenless colonies. In contrast, variation in oxidative stress resistance and gene expression was more strongly associated with division of labour (inside versus outside workers) than with queen number. Queens of this species exhibited remarkable physiological stability across age classes. **Chapter 2** uses the clonal raider ant *Ooceraea biroi* to experimentally test the causal relationship between reproduction and lifespan. Experimental suppression of reproduction significantly reduced worker survival, whereas continuous or naturally cycling reproduction was associated with increased longevity. Reproductive workers showed elevated expression of genes involved in antioxidant defence, immune function, and DNA repair, providing direct experimental evidence for a reversal of the classical longevity/fecundity trade-off in social insects. **Chapter 3** investigates the effects of queen loss in *Messor capitatus*, a monogynous species with potentially totipotent workers. Although queen removal induced ovarian activation, it did not consistently extend worker lifespan. Instead, colony size emerged as a key determinant of survival, indicating that life-history responses to reproduction are strongly context dependent. **Chapter 4** focuses on *Hypoponera opacior,* a species with obligately sterile workers. In this system, queen absence had no effect on worker lifespan, suggesting that reproductive potential is a prerequisite for lifespan plasticity following queen loss. **Chapter 5** examines the genetic basis of alternative reproductive strategies in *H. opacior*. Genome-wide analyses revealed no evidence for supergene-linked determination of reproductive morphs, instead supporting a model of plastic, environmentally mediated regulation of reproductive morphology.

In conclusion, this dissertation demonstrates that ageing and lifespan in ants are governed by the interplay between social organisation, reproductive status, and physiological plasticity. The findings show that the classical trade-off between reproduction and longevity can be reversed when reproduction occurs within a socially protected context, providing new insights into the evolutionary and mechanistic foundations of ageing.

General Introduction

The evolutionary puzzle of ageing

Ageing is commonly defined as the intrinsic, progressive decline in an organism's physiological function, leading to reduced fertility and increased mortality over time (López-Otín et al., 2013; Rose, 1990). Despite its obvious costs to survival and reproduction, this extremely diverse process has persisted across the tree of life (Cohen, 2018; Jones et al., 2014). With Aristotle possibly being the first to raise theoretical interest in the causes of ageing over 2,000 years ago, its process captivated scientists for centuries, representing one of the longest-standing evolutionary paradoxes (de Magalhães, 2024; Woodcox, 2018). Nonetheless, because ageing is a multifaceted process that unfolds across tissues, timescales, and ecological contexts rather than reflecting a single underlying mechanism, its study remains one of biology's substantial challenges(de Magalhães, 2024). Consequently, central questions remain to date: which mechanisms drive ageing, and how can its costs be balanced or compensated in evolutionary terms?

Over the last 100 years, advances on many fronts have converged into a broad explanatory framework for the biology of ageing. A central principle emerges from evolutionary theories that have been particularly influential, as they address why natural selection allows ageing to persist despite its costs. The "*mutation accumulation theory"* (Medawar, 1952) posits that with age, the force of natural selection declines and consequently permits deleterious mutations to accumulate. In turn, this accumulation progressively increases damage and leads to senescence. An extension of this theory was provided by the "*antagonistic pleiotropy theory*" (Williams, 1957), which proposes that genes with beneficial effects early in life may have detrimental effects later. Yet, despite promoting senescence, they are still favoured by selection as they lead to more offspring. The "*disposable soma theory*" (Kirkwood, 2017, 1977; Kirkwood and Austad, 2000; Kirkwood and Holliday, 1997) introduced a resource allocation framework: since resources are limited, organisms face trade-offs between investing in reproduction (germline) or in somatic maintenance. Thus, investment in the germline comes at the cost of the soma, leading to ageing over time. Holliday's "*energy allocation hypothesis*" (1989) further linked this framework to earlier theories on the impact of caloric restriction

(Barrows and Kokkonen, 2018; McCay et al., 1935; Weindruch and Walford, 1988), suggesting that under limited resources, organisms shift towards enhanced maintenance and repair, thereby slowing ageing (Holliday, 1989).

These evolutionary perspectives were further complemented by mechanistic theories. The "*rate of living hypothesis"* (Pearl, 1928) proposed that organisms with higher metabolic rates accumulate molecular damage more quickly, leading to shorter lifespans. This hypothesis was further supported as larger animals with lower mass-specific metabolic rates typically live longer (Gillooly et al., 2005; Speakman, 2005). However, this assumption was challenged by studies showing that metabolic rate cannot solely predict lifespan across taxa (Hulbert et al., 2004; Stark et al., 2020). At the cellular level, the concept of replicative senescence, caused by telomere shortening, highlighted limits to cell proliferation and organismal lifespan (Bodnar et al., 1998; Hayflick and Moorhead, 1961; Olovnikov, 1996). Moreover, **r**eactive oxygen species (ROS), by-products of mitochondrial respiration, are core elements of the "*free radical theory*" and considered a main source of molecular damage (*i.e.,* oxidative damage) (Barja, 2004; Harman, 1956). They further link metabolic and damage-based theories and contribute to telomere erosion (Finkel and Holbrook, 2000; Sohal and Weindruch, 1996; von Zglinicki et al., 2001, 1995). However, these mechanistic processes do not contradict evolutionary theories of ageing. Instead, they represent physiological pathways through which evolutionary trade-offs between reproduction, maintenance, and survival may be realised. Because selection acts on fitness rather than on longevity per se, such mechanisms may persist when their mitigation would compromise other fitness components, or when their costs are expressed primarily late in life.

Beyond metabolism and the accumulation of molecular damage, ageing is also shaped by the costs associated with physiological stress responses. Maintaining homeostasis in the face of environmental challenges requires the coordinated activation of multiple defence and repair systems, many of which impose energetic demands or generate collateral damage. Immune function provides a well-studied example of such trade-offs. While robust immunity enhances survival by preventing infection, immune responses such as inflammatory processes are energetically costly and can induce

oxidative stress or self-damage if not properly regulated (Deveale et al., 2004; Fabian et al., 2021; Lochmiller and Deerenberg, 2000). Numerous studies across different taxa illustrate the link between immune activity and longevity. For example, in the mealworm beetle *Tenebrio molitor* early-life immune challenges reduce subsequent reproductive output or accelerate the decline in immune function. These effects do not necessarily extend lifespan but impose significant costs on key fitness components (Jehan et al., 2022). In *Drosophila*, overexpression of immune pathways shortens lifespan (Deveale et al., 2004; Libert et al., 2006), and regulation of immunity influences longevity trade-offs in *Caenorhabditis elegans* (Naim et al., 2021; Otarigho and Aballay, 2021). These observations strengthen the idea that longevity does not necessarily demand suppressed immunity, but rather a well-tuned immune system that avoids chronic inflammation or immunopathology. Thus, longevity is not achieved by suppressing stress responses such as immunity, but by refining their deployment to balance protection with their energetic and physiological costs. The optimal balance between defence, maintenance, and reproduction is therefore expected to vary across species, environments, and life stages (Deveale et al., 2004; Jehan et al., 2022).

As in many scientific fields, ageing research has experienced paradigm shifts (Kuhn and Hacking, 1970). Central assumptions, such as the role of ROS were increasingly questioned (de Magalhães and Church, 2006; Gems and Doonan, 2009; Pérez et al., 2009). This, in turn, not only weakened the assumption that damage accumulation plays a pivotal role in ageing but also raised doubt on association frameworks such as the disposable soma theory (Blagosklonny, 2010; Maklakov and Chapman, 2019). Instead, new drivers of ageing gained prominence: the nutrient-sensing pathways “(mammalian) target of rapamycin” (mTOR) and “insulin/insulin-like growth factor 1 signalling” (IIS) (Gems and de Magalhães, 2021). These two pathways are highly conserved, tightly integrated and are more directly linked to reproduction, growth and development rather than somatic maintenance (Blagosklonny, 2008; de Magalhães and Church, 2005; Efeyan et al., 2015; Gems and Partridge, 2013; Kenyon, 2005). For instance, the upstream modulation of IIS in insects impacts the downstream production of juvenile hormone (JH) and ecdysteroids which are involved in development, reproduction and metabolic regulation (Li et al., 2019). Manipulation of JH biosynthesis

has profound effects on ageing and its inhibition extends lifespan in multiple insect species (Herman and Tatar, 2002; Hodkova, 2008). Moreover, reduced JH titres have been shown to prolong life in *Drosophila melanogaster* and plays a pivotal role in reproductive investment (Yamamoto et al., 2013). It stimulates the production of vitellogenins, which are female-specific lipoproteins that are essential for egg production (Sheng et al., 2011; Wyatt and Davey, 1996; Zhu et al., 2020).

However, this reproductive role creates a conflict: upregulation of the IIS pathway promotes fecundity at the cost of somatic maintenance, reflecting classic pleiotropy (Flatt and Kawecki, 2007; Toivonen and Partridge, 2009). In this context, JH contributes to the balance between reproduction and survival. The TOR pathway, similarly, links nutrition to lifespan regulation. Inhibition of TOR extends lifespan in organisms ranging from yeast to mammals (Fontana et al., 2010; Hansen et al., 2008; Harrison et al., 2009; Kapahi et al., 2004; Powers et al., 2006). This extension has been attributed to enhanced autophagy, a self-repair mechanism that recycles damaged cellular components and maintains proteostasis (Bjedov et al., 2010; Madeo et al., 2015; Rubinsztein et al., 2011). However, excess protein intake or amino acid imbalance can overstimulate TOR, suppress autophagy, and accelerate ageing (Simpson and Raubenheimer, 2009). Thus, TOR has been described as a key modulator of ageing that acts as a mediator of the beneficial actions of dietary restrictions due to its ability to integrate nutritional, hormonal, and stress-related signals (de Magalhaes et al., 2012; Johnson et al., 2013).

Recent perspectives frame ageing as a multidimensional framework involving a complex interplay between environmental factors, genetic pathways and life-history traits (Li et al., 2023; Figure 1). While molecular, cellular and metabolic mechanisms such as IIS, TOR, DNA repair, fatty acid metabolism, inflammation and cell-cycle have been studied extensively (Kolora et al., 2021; Kowalczyk et al., 2020; Ma and Gladyshev, 2017; Singh et al., 2019; Tian et al., 2017; Zhou et al., 2020), ageing also reflects how organisms allocate resources across competing life-history demands (Maklakov and Chapman, 2019). In particular, reproduction and somatic maintenance represent fundamental trade-offs, as investment in fecundity can come at the cost of lifespan, and vice versa (Kirkwood, 2017; Maklakov and Chapman, 2019). This trade-off framework

provides a crucial link between evolutionary theories of ageing and proximate mechanisms. These perspectives show that ageing is not driven by a single cause, but by an evolving balance between survival, reproduction, and body repair. Understanding how these trade-offs are managed across species may hold the key to modulating ageing itself.

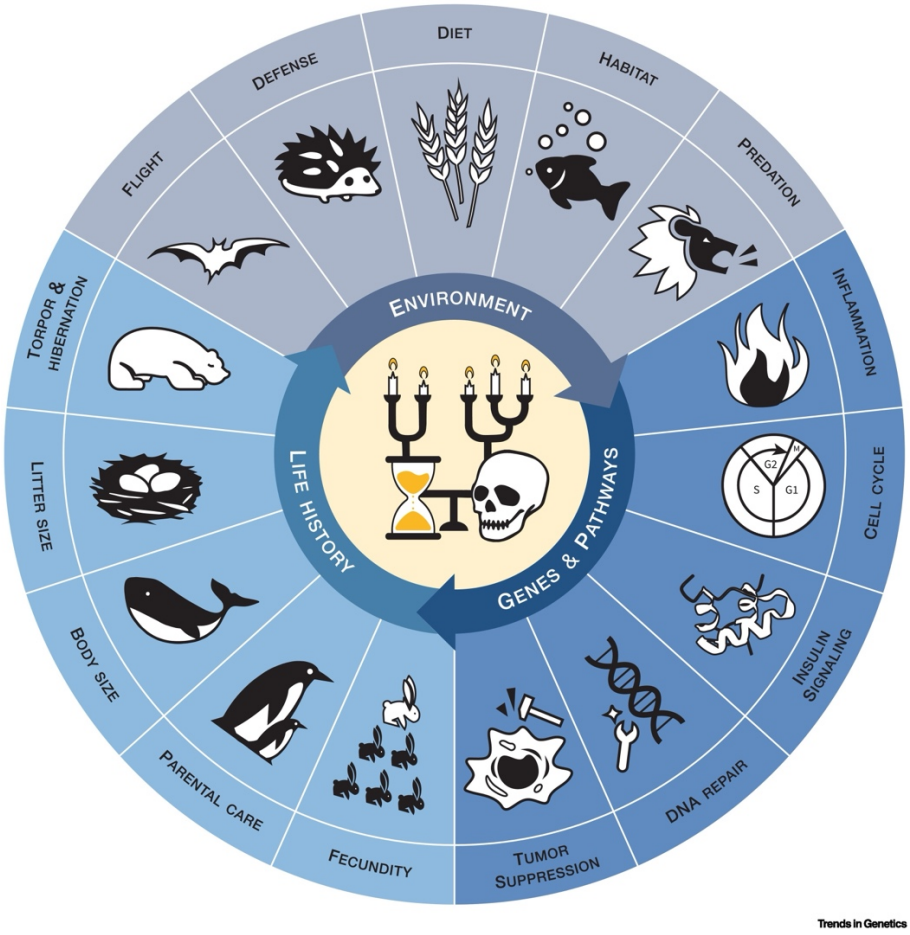

Figure 1: Key figure from Li et al. (2023) illustrating evolutionary dynamics underlying ageing and longevity.

Ageing and lifespan are shaped by the interaction between genetic pathways, life-history traits and environmental factors. Illustrated here are representative elements for each domain that have been associated with variation in ageing and longevity. Differences in lifespan are symbolized by candle height and their evolutionary context (Li et al., 2023).

Lessons from eusocial insects

Most mechanistic insights into the molecular regulation of ageing stem from rather short-lived model organisms such as yeast, worms, and flies (Kenyon, 2010; Partridge and Gems, 2002). While these systems offer genetic tractability and fast generation times, they may not capture the full diversity of ageing strategies in nature (Austad, 2009; Partridge and Gems, 2002). In contrast, eusocial insects such as ants, some bees, and termites have evolved extraordinary variation in caste-specific lifespan, making them compelling models for ageing research (Parker, 2010; Jemielity et al., 2005; Keller and Jemielity, 2006).

Eusociality is defined by three hallmark traits: cooperative brood care, overlapping generations, and reproductive division of labour among females (Crespi and Yanega, 1995). In insects, eusociality has evolved multiple times independently and is widely regarded as a "*major evolutionary transition*" (MET; Bourke, 2011; Szathmáry and Smith, 1995), in which selection acts not only at the individual level but also at the colony level. Social insect colonies are often described as "superorganisms**"** in which reproductives function as the germline and non-reproductive workers as the soma (Boomsma and Gawne, 2018; Wheeler, 1911). The consequences for life history are profound. Eusocial insect colonies are composed of a reproductive caste: queens (and kings in termites), and a non-reproductive caste: the workers (Wilson, 1971). In this setting, queens monopolize reproduction, while workers engage in various other tasks such as brood care, foraging and nest defence (Hölldobler and Wilson, 1990). Both castes differ tremendously in morphology, physiology, behaviour, fecundity and exhibit extreme lifespan differences (Hölldobler and Wilson, 1990; Keller and Genoud, 1997; Wilson, 1971). Importantly, these caste-specific lifespan differences are not merely curiosities. Eusocial reproductives provide rare examples of organisms that combine extremely high fecundity with extraordinary longevity. Workers, in contrast, typically conform to shorter lifespans but retain a spectrum of reproductive potential across species, from obligate sterility to totipotency (Bourke, 1988; Heinze and Schrempf, 2008). Together, this diversity creates a rich comparative framework for testing both proximate and ultimate theories of ageing.

Queens: long-lived, yet highly fecund

Despite being the most fertile individuals in the colony, social insect queens may live decades while typically non-reproductive workers survive only weeks or months (Corona et al., 2016; Keller and Genoud, 1997; Keller and Jemielity, 2006). Therefore, social insect queens challenge the conventional trade-off between longevity and fecundity by exhibiting both, an extraordinarily long lifespan and very high fecundity. At the same time, even across species queens exhibit a variation in lifespan and reproductive investment. This is especially evident in ant queens: In monogynous systems (one queen per colony), single queens can live for up to 30 years (*e.g., Lasius niger*; Hölldobler and Wilson, 1990). In polygynous species (multiple queens per colony), however, individual queens often live shorter lives, as shown in *Cardiocondyla obscurior (*six months; Oettler and Schrempf, 2016)*, Solenopsis invicta (*one to three years; Goodisman and Ross, 1999), and *Linepithema humile* (one year; Keller, 1998; Reuter et al., 2001; Schrempf et al., 2011). This difference in longevity is closely linked to colony founding strategies. In monogynous species, queens often establish colonies independently and are well-provisioned (*e.g., Lasius niger*; Janet, 1907). The death of the sole queen usually results in colony demise and, as a consequence, selection against queen mortality remains strong throughout the queen's life. In contrast, queens in polygynous species often mate locally and remain within their natal nest (*e.g., Formica selysi*; Keller, 1995; *Tapinoma magnum;* Lenhart et al., 2025; Seifert et al., 2017), where the loss of an individual queen can be buffered by the presence of co-reproductive queens, reducing the fitness cost of queen death at the colony level. Yet, even in these "short-lived" species, queens consistently outlive their non-reproductive workers (Oettler and Schrempf, 2016; Porter, 1988; Tschinkel, 1988), highlighting that reproductive status consistently confers substantial longevity advantages across lineages.

Growing evidence suggests that the genetic architecture underlying social organisation may also shape the extent to which life-history plasticity is possible. In most eusocial insects, queens and workers share a largely identical genetic background, and pronounced differences in behaviour, physiology, and lifespan arise through developmental and physiological plasticity rather than genetic divergence. Nevertheless, in several ant lineages, alternative social organisation such as monogyny

and polygyny are controlled by supergenes, large non-recombining genomic regions that link multiple traits related to reproduction, dispersal, and behaviour (Braim, 2015; Brelsford et al., 2020; De Gasperin et al., 2025; Errbii et al., 2022; Kay et al., 2022; Lagunas-Robles et al., 2021; Lajmi et al., 2025; Pracana et al., 2017; Scarparo et al., 2023; Sigeman et al., 2025; Trible et al., 2023; Wang et al., 2013). By genetically coupling social structure with suites of behavioural and physiological traits, supergenes may stabilise particular reproductive strategies and, in doing so, constrain life-history variation. These findings raise the possibility that differences in ageing and lifespan among social insects reflect not only variation in reproductive physiology and social environment, but also deeper constraints imposed by genomic architecture. Understanding how ageing evolves in eusocial systems therefore requires integrating mechanisms of physiological plasticity with the genetic factors that canalise or restrict alternative social and reproductive strategies.

Despite potential constraints imposed by genetic architecture, ageing trajectories in social insect queens remain highly variable. In some species, queen longevity and lifetime reproductive output are positively correlated (Schrempf et al., 2017), suggesting sustained reproductive performance across much of the lifespan. Consistent with this, termite queens show little evidence of senescence until shortly before death(Monroy Kuhn et al., 2021). However, reproductive senescence is not absent in social insects. Honeybee and bumblebee queens often show declining fertility with age, the latter accompanied by increasing worker aggression (Amsalem et al., 2014; Tarpy et al., 2000; Woyke, 1971). In short-lived *C. obscurior* queens, egg-laying rates decline with age, but queens compensate by producing a higher proportion of sexual offspring late in life (Jaimes-Niño et al., 2022), while in long-lived *Temnothorax rugatulus queens*, tissue-specific ageing patterns suggest a shift from immune to antioxidant investment as queens age (Negroni et al., 2019). This mirrors findings in model organisms such as *Drosophila*, *C. elegans*, and even humans, where ageing manifests differently across tissues (Tain et al., 2021; Wang et al., 2022; Yamamoto et al., 2022). Such variation suggests that queens do experience senescence, but its onset and expression may be postponed, tissue-dependent, or masked by colony-level buffering.

In recent years, ageing research in social insects has increasingly shifted toward uncovering the molecular mechanisms behind their unique ageing trajectories as classical proximate theories, such as the free radical theory of ageing, appear to rather inconsistently explain the absence of a fecundity/longevity trade-off: Long-lived reproductives do not consistently show elevated antioxidant activity (Parker, 2010; Kramer et al., 2021). Also, in *Lasius niger*, no caste-specific differences in telomere length have been detected (Jemielity et al., 2007). Moreover, increasing evidence underscores the importance of the nutrient-sensing pathways IIS, TOR, JH signalling as well as vitellogenin which has undergone repeated duplication and subfunctionalization in social lineages and not only contributes to fecundity but also to caste differentiation, behaviour, oxidative stress resistance, and other anti-ageing processes (Chandra et al., 2018; Corona et al., 2013, 2007; Feldmeyer et al., 2014; Parker, 2010; Kohlmeier et al., 2019; Libbrecht et al., 2018, 2013; Seehuus et al., 2006; Weger and Rittschof, 2024; Yan et al., 2022). Consequently, a recent comparative work across ants, bees and termites has begun to synthesize these insights. Korb et al. (2021) proposed the TI-J-LiFe (TOR/IIS–JH–Lifespan and Fecundity) framework (Figure 2), which highlights the downstream role of endocrine and reproductive regulators such as JH, vitellogenins, and antioxidant/immune pathways (Korb et al., 2021). Comparative transcriptomics revealed that ageing and fecundity are underpinned by more species-specific mechanisms than previously assumed, yet converging around networks of JH, vitellogenin, immunity, and oxidative stress responses (Korb et al., 2021). While many functional aspects remain unresolved, especially in non-model organisms, the exceptional phenotypic plasticity in lifespan and reproduction found in both queens and workers provides a powerful opportunity to dissect how the fecundity/longevity trade-off has been reversed in insect societies.

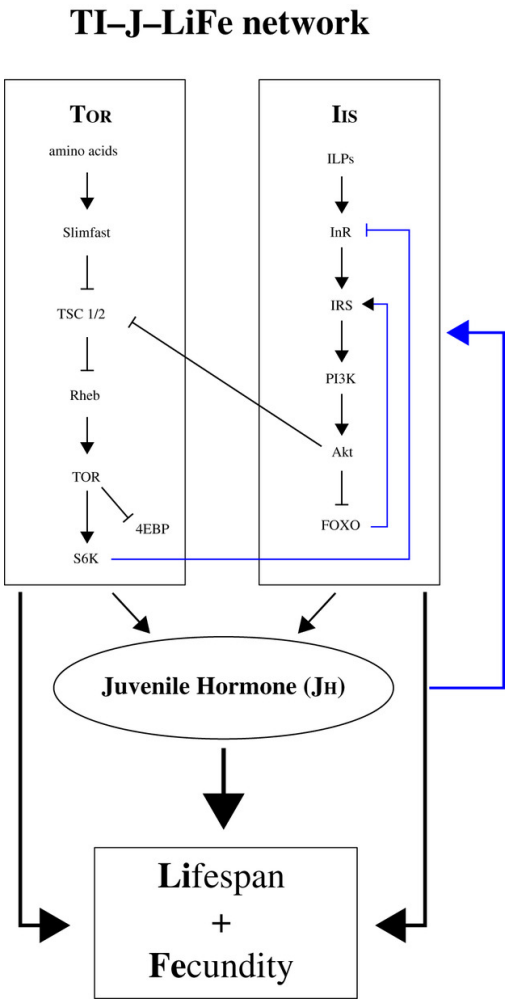

Figure 2: The 'TI–J–LiFe' network from Korb et al. (2021).

The TI–J–LiFe network integrates key hormonal and nutrient-sensing pathways that jointly regulate insect lifespan and fecundity. It encompasses the TOR (Target of Rapamycin) and IIS (Insulin/Insulin-like Growth Factor 1 Signaling) pathways, the Juvenile Hormone (JH), and their downstream targets involved in somatic maintenance (*e.g.,* immunity, oxidative stress resistance) and reproductive physiology (*e.g.*, vitellogenins and yolk proteins). Together, these interconnected pathways form a major regulatory circuit shaping the trade-off between longevity and reproduction in insects. The core components and feedback loops illustrated here are primarily based on experimental evidence from *Drosophila melanogaster* (Korb et al., 2021).

Yet, the extraordinary longevity that makes social insect queens so remarkable also poses a practical challenge for ageing research. Even a lifespan of around one year, as seen in relatively short-lived queens, represents a very long timeframe for experimental work, while the lifespans of long-lived queens may exceed the duration of entire research projects. Consequently, most current insights into queen ageing come from comparative and molecular approaches that capture specific stages rather than

full lifetimes (Korb, 2016). Moreover, sociality itself may confound interpretation: queens may appear exempt from classical trade-offs not because costs vanish, but because they are shifted to workers or buffered at the colony level. Social living buffers individuals against stressors in ways unavailable to solitary species (shared thermoregulation, food redistribution, or task reallocation) and may further mitigate classic life-history trade-offs (Koto et al., 2023; Walton et al., 2024). Nevertheless, evidence from cooperative breeders indicates that reproductive costs are not universally eliminated by sociality and may still be detectable under certain conditions (Sudyka et al., 2019), suggesting that social buffering can mitigate but does not necessarily abolish life-history costs. Hence, social life itself can act as a form of stress protection, helping explain how high fecundity coexists with long life in reproductives (Walton et al., 2024). At the same time, responses to queen loss or isolation vary widely among lineages, emphasizing that ecological context and social architecture shape ageing trajectories (Koto et al., 2023; Lenhart et al., 2025; Lopes et al., 2020; Majoe et al., 2024, 2021; Negroni et al., 2021b). Taken together, these advances refine the view that "queens live long because they reproduce" into a more integrated picture: eusocial longevity emerges from multilevel regulation including nutrient-sensing and endocrine rewiring, epigenetic control, social immunity, trophallactic signalling, and demography, collectively reshaping reproductive costs and the selection shadow at the colony level (Blacher et al., 2017; Friedman et al., 2020; Sieber et al., 2021). Thus, while previous studies provided critical foundations for our understanding of queen ageing, the life-history of social insects also highlights the need for complementary approaches to fully resolve how reproduction and longevity are linked in these extraordinary insects (Korb, 2016).

Workers: typically short-lived and non-reproductive

While obligate eusociality is often defined by strict reproductive division of labour where queens monopolize reproduction and workers are functionally sterile (Bourke, 2011; Hamilton, 1964; Queller and Strassmann, 1998), this definition does not capture the full diversity observed across social insects. Worker reproductive capacity varies widely among lineages, ranging from complete sterility to full reproductive totipotency. At one end of this spectrum is obligate worker sterility which is marked by the irreversible loss of reproductive ability. This pattern has been documented in at least six genera of

Ponerinae (*e.g., Hypoponera opacior*; Foitzik et al., 2002) and eight genera of Myrmicinae (*e.g., Monomorium pharaonis;* Hölldobler and Wilson, 1990; Peeters and Ito, 2015; Warner et al., 2017). At the opposite extreme, some ants exhibit worker totipotency through thelytokous parthenogenesis, a form of asexual reproduction in which females may develop from unfertilized eggs (Rabeling and Kronauer, 2012). This ability enables workers to produce female offspring and, in certain species, even replace the queen entirely. This phenomenon has been reported in at least 20 ant species, including *Cataglyphis cursor* and *Cataglyphis sabulosa* (Pearcy et al., 2006, 2004; Rabeling and Kronauer, 2012; Timmermans et al., 2008). In many cases, thelytoky occurs only rarely and typically in orphaned colonies, but in others, it has led to the secondary loss of the queen caste (Rabeling and Kronauer, 2012; Kronauer et al., 2013). Adding to this diversity, workers in several species are even capable of sexual reproduction via so-called gamergates (Carmona-Aldana et al., 2024). Such workers possess functional spermathecae and can mate, store sperm and as a result, lay haploid (male) and diploid (female) eggs. This phenomenon is most found in species with low dimorphism between castes. For example, in *Platythyrea punctata* both types of worker reproduction may occur: thelytokous parthenogenesis and sexual reproduction (Brunner et al., 2009; Schilder et al., 1999).

However, in most social insect species, especially in ants, workers fall between these extremes and are considered facultatively sterile. In those species, workers retain a limited reproductive potential (Bourke, 1988; Heinze et al., 1997; Heinze and Schrempf, 2008; Heinze and Tsuji, 1995; Ratnieks et al., 2006) and, under certain condition, can produce haploid eggs via arrhenotokous parthenogenesis (Bourke, 1988; Crozier and Pamilo, 1996; Hammond and Keller, 2004). Importantly, not every worker-laid egg is viable, as workers may produce non-viable nutrient-rich trophic eggs (Peeters, 1991). Although workers in many social insect species retain functional ovaries (Bourke, 1988), their reproductive activity is typically suppressed in the presence of a queen. This suppression may occur through self-restraint or active policing by nestmates (Dijkstra et al., 2010; Dijkstra and Boomsma, 2006; Holman et al., 2010; Wenseleers and Ratnieks, 2006), but also through queen pheromones, often mediated by cuticular hydrocarbons (CHCs) (D'Ettorre et al., 2004; Hölldobler and Wilson, 1983; Sharma et al., 2015; Zeng,

2023). Queens produce distinctive blends of CHCs that function as fertility signals and directly inhibit worker ovarian activation (Holman et al., 2010; Van Oystaeyen et al., 2014). For instance, in *L. niger* and *Cataglyphis iberica*, experimental application of synthetic queen pheromones suppressed worker ovary development (Holman et al., 2010; Van Oystaeyen et al., 2014).

If the queen is absent, these workers activate their ovaries, fight over reproductive dominance, and start reproduction (Blacher et al., 2010; Boomsma, 2009; Heinze et al., 1997; Yagound et al., 2014). This facultative shift has repeatedly been linked to changes in physiology, immunity and lifespan. Reproductive workers have better developed ovaries, exhibit an increased oxidative stress resistance and often live longer than their non-reproductive counterparts (Blacher et al., 2017; Dixon et al., 2014; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021). At the molecular level, those workers often exhibit altered brain and fat body gene expression, reflecting changes in metabolic, immune and ageing pathways (Choppin et al., 2021; Korb et al., 2021; Lenhart et al., 2025; Negroni et al., 2021b, 2021a). Similar patterns were observed in totipotent workers where worker reproductive activity is consistently associated with prolonged lifespans. For instance, in the ant *Harpegnathos saltator*, workers that acquire reproductive status as gamergates outlive their non-reproductive nestmates by up to a factor of five (Ghaninia et al., 2017; Glastad et al., 2023; Yan et al., 2022). In *P. punctata*, dominant individuals that monopolize egg laying have approximately double the lifespan of their subordinate sisters (Hartmann and Heinze, 2003; Hartmann et al., 2020; Matte et al., 2024). Importantly, in these systems reproductive workers typically adopt a queen-like social role: they remain inside the nest, receive care from nestmates, and are largely shielded from foraging and other high-risk tasks (Hartmann and Heinze, 2003). Thus, these findings suggest that reproduction, when embedded within a protected social role, can promote longevity in social insect workers, inverting the conventional trade-off between fecundity and lifespan. Moreover, the retention of reproductive potential in workers implies that the fitness consequences of survival depend on social context. When workers can gain direct fitness after queen loss, selection may favour increased investment in somatic maintenance. This effect is expected to be strongest in species with totipotent workers, where reproductive individuals can monopolize reproduction,

adopt protected, nest-bound roles, and maintain reproductive status over extended periods. Conversely, in species where worker reproduction is absent or severely constrained, selection for extended worker lifespan is expected to remain weak, even in the absence of a queen.

Besides species-specific differences, worker reproductive ability can be influenced by age, task and environment. Inside workers, often younger and physiologically richer in fat reserves, live longer than outside foragers, whose risky duties expose them to higher mortality (Choppin et al., 2021; Kohlmeier et al., 2017; Lenhart et al., 2025; Negroni et al., 2021b). Most often, it is the younger inside worker that may activate their ovaries and start reproduction over an older outside worker. This pattern reflects the principle of age polyethism, where younger, more valuable individuals remain protected in the nest, and older workers transition to risky outside tasks (Blanchard et al., 2000; Chapuisat and Keller, 2002; Hartmann et al., 2019). Physiologically, these older foragers tend to show reduced fat stores and regressed ovaries (Bernadou et al., 2020; Lenhart et al., 2025), consistent with declining residual lifespan. While task-switching can occur depending on colony needs, task, age, and reproductive potential remain strongly correlated in most ant societies (Iwasa and Yamaguchi, 2020; Quque et al., 2023). Environmental and social factors further modulate worker reproduction (Carmona-Aldana et al., 2024; Haight and Liebig, 2025; Heinze and Giehr, 2021).

Crucially, this plasticity makes workers powerful models for ageing research. Unlike queens, whose long lifespans pose logistical challenges for experimental studies, workers allow direct testing of how reproductive activation impacts physiology, oxidative stress, immunity, and survival (Blacher et al., 2017; Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021). Their diversity captures a wide spectrum of reproductive strategies, while their within-colony variation in age, task, and reproductive state provides a natural experimental framework. Worker ants, thus, offer an opportunity to disentangle the proximate and evolutionary links between reproduction and lifespan, complementing queen-focused studies and broadening our understanding of ageing in social organisms.

Objectives

In my dissertation, I investigate the interplay between ageing, reproduction, and social organisation in ants, with a focus on how caste and reproductive status shape lifespan. Using four ant species with extremely divergent social and reproductive systems, I examined how factors such as queen presence / number, worker reproductive potential, and caste-specific physiology contribute to ageing trajectories. In **Chapter 1** I used the invasive, highly polygynous ant *Tapinoma magnum* to investigate how queen number and age shape ageing- and reproduction-related traits. In facultatively sterile workers, I assessed survival, ovarian development, oxidative stress resistance and fat body gene expression, while in queens I compared age-related fecundity and transcriptomic profiles. I found that workers survived the longest in single-queen colonies and developed more oocytes compared to two-queen colonies, while queenless workers showed the highest mortality. Presumably younger inside workers exhibited greater oxidative stress resistance and upregulated antioxidant genes than older outside workers. By contrast, presumably short-lived queens displayed stable fecundity and transcriptomic profiles with age. These findings reveal that social context and division of labour strongly modulate survival, reproduction, and gene expression in workers, whereas queens of *T. magnum* remain physiologically robust (Lenhart et al., 2025). This chapter provides novel insights into how social structure modulates physiology and gene expression in a strictly polygynous species.

While *T. magnum* represents a highly polygynous system where workers are incapable of active reproduction, the clonal raider ant *Ooceraea biroi* offers a striking contrast. In this species, the queen caste is completely lost and all workers reproduce synchronously via thelytokous parthenogenesis – a trait that can be experimentally manipulated (Ravary et al., 2006; Teseo et al., 2013). In **Chapter 2**, I aimed to experimentally test the causal link between reproduction and lifespan. By experimentally manipulating same-age and genetically identical workers to either reproduce continuously, remain in a non-reproductive state, or alternate naturally between phases, I could disentangle reproductive activity from confounding factors such as age, morphology, and genetic background. The results revealed that suppressing

reproduction reduced worker survival, while continuous reproduction or natural cycling maintained longevity. Transcriptomic analyses of brain and fat body tissues further revealed that reproductive activity was associated with the maintenance or upregulation of genes involved in antioxidant defence, detoxification, immunity, and DNA repair – pathways central to cellular homeostasis and ageing. Notably, as workers aged, those that were experimentally induced to reproduce maintained or even increased the expression of these protective genes compared to non-reproductive workers that were prevented from egg laying. These findings provide direct experimental evidence for the reversal of the longevity/fecundity trade-off in social insects and highlight candidate molecular mechanisms linking reproductive activation to extended lifespan.

In **Chapter 3**, I investigated how queen loss influences worker survival, ovarian development, and reproductive output in the European seed-harvesting ant *Messor capitatus* – a species where workers were previously reported to reproduce via thelytokous parthenogenesis in the absence of the queen (Grasso et al., 2000). Here, I aimed to investigate whether totipotent worker reproduction alters worker survival. Contrary to findings in other monogynous ant species, queen removal did not generally extend worker lifespan, although it stimulated ovarian development and oviposition. Worker survival was instead strongly influenced by sub-colony size, with overall survival differing among size categories. Moreover, the effect of queen loss on survival varied across sub-colony sizes: while workers in large queenless sub-colonies survived better than those in queenright counterparts, queen loss was associated with reduced survival in medium- and small-sized sub-colonies. Despite increased ovarian development, only a minority of queenless colonies produced eggs, but none progressed to adulthood, providing no clear evidence of successful thelytokous reproduction under laboratory conditions. These findings indicate that although queen loss in *M. capitatus* induces physiological readiness for reproduction, this response does not translate into increased worker longevity or effective queen replacement.

Chapter 4 shifts focus to a species where worker reproductive potential is entirely absent. In the neotropical ant *Hypoponera opacior*, workers are obligately sterile, lacking the ability to activate ovaries under any condition (Foitzik et al., 2002). This system

provided a rare opportunity to test whether reproductive capacity itself is essential for lifespan extension after queen loss. By experimentally comparing worker survival in queenless and queenright colonies, we found no evidence that queen absence prolonged or shortened worker lifespan. Unlike species where workers respond to orphaning with ovarian activation, oxidative stress resistance, and transcriptional changes, *H. opacior* workers remained short-lived regardless of queen presence. These results highlight that reproductive potential appears a key determinant of lifespan plasticity in social insects, and that obligate sterility seemingly eliminates this classic link between reproduction and longevity.

While the preceding chapters highlight how ageing and lifespan plasticity in ants are shaped by reproductive physiology and social context, they also raise a broader question: to what extent are these reproductive strategies themselves developmentally flexible versus genetically fixed? If reproductive roles are developmentally plastic, social context can rapidly reshape life-history trajectories. However, if reproductive strategies are genetically canalised, such constraints may limit the scope for plastic responses in physiology, behaviour, and ageing. In several ant lineages, alternative social organisations and reproductive strategies are controlled by supergenes. By genetically coupling social structure with coordinated behavioural, physiological, and life-history traits, supergenes have the potential to stabilise particular reproductive strategies across generations and, in doing so, constrain variation in ageing trajectories. Understanding whether variation in lifespan and reproductive roles arises from flexible developmental responses or from genetically fixed architectures is therefore central to understanding how ageing evolves in eusocial systems.

This distinction is particularly relevant for species exhibiting discrete alternative reproductive morphs, where sharp phenotypic differences may reflect either genetic determination or plastic developmental pathways. **Chapter 5** addresses this by examining the genetic architecture underlying reproductive polymorphism in *H. opacior*, a species with pronounced alternative reproductive morphs in both sexes (ergatoid and winged queens and males) that are associated with different colony organisations (Foitzik et al., 2011a, 2010, 2002; Kureck et al., 2012). By testing whether these morphs

are underpinned by a supergene architecture or instead arise through plastic mechanisms, Chapter 5 extends the thesis framework from physiological and social regulation of ageing to the genetic constraints that may shape reproductive diversity and life-history variation. Using whole-genome resequencing of individuals representing alternative morphs, I found no evidence for large-scale genomic differentiation or supergene architecture. Instead, the results suggest that the expression of ergatoid and winged morphs likely arises through phenotypic plasticity, environmentally sensitive developmental switches, or polygenic regulation. These findings highlight the importance of plastic and ecological mechanisms in generating reproductive diversity.

1

Chapter 1

Worker survival and egg production– but not transcriptional activity– respond to queen number in the highly polygynous, invasive ant *Tapinoma magnum*

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Published as: Lenhart, A., Majoe, M., Selvi, S., Colgan, T.J., Libbrecht, R. and Foitzik, S. (2025), Worker Survival and Egg Production—But Not Transcriptional Activity—Respond to Queen Number in the Highly Polygynous, Invasive Ant *Tapinoma magnum*. *Mol Ecol,* 34: e17679. https://doi.org/10.1111/mec.17679

Abstract

In social animals, reproductive activity and ageing are influenced by group composition. In monogynous (single-queen) insect societies, queen presence affects worker fecundity and longevity, but less is known about worker responses to queen number variation in polygynous (multi-queen) species or how queens age in these systems. We created queenless, one-queen, and two-queen colonies of the invasive, polygynous ant *Tapinoma magnum* to examine the effect of queen number on worker survival, ovary and oocyte development, oxidative stress resistance, and fat body gene expression. We also compared the fecundity and brain and fat body transcriptomes between young and old queens. Queenless workers experienced the highest mortality, contrasting with monogynous species, where queen removal typically extends lifespan. Workers lived longer and had more developing oocytes in their ovaries in single-queen than in two-queen colonies. Queen number did not directly affect oxidative stress resistance or fat body gene expression, though its effect on the latter differed between inside and outside workers. Furthermore, inside— likely younger— workers produced more oocytes, showed higher oxidative stress resistance, and upregulated antioxidant genes compared to outside— likely older— workers. Minimal shifts in fecundity and gene expression of differently aged queens indicated their physiological stability. Our research highlights distinct caste- and tissue-specific responses to varying queen numbers in workers of a highly polygynous species

Keywords: reproductive division of labour, invasive species, polygyny, life history, ageing, gene expression

Chapter 1

Introduction

A fundamental tenet of life history theory predicts that organisms are confronted with trade-offs. These trade-offs can be driven by resource allocation, e.g., organisms must decide whether to invest more resources into body maintenance or reproduction. They can also evolve as a consequence of suboptimal gene regulatory networks for both reproduction and body maintenance (Kirkwood, 2017; Maklakov and Chapman, 2019). Ageing – the inevitable intrinsic deterioration with age, limits the reproductive potential of an organism, often resulting in a negative correlation between longevity and fecundity (Chapuisat and Keller, 2002; Partridge and Harvey, 1988). As organisms age, the accumulation of molecular and cellular damage leads to functional decline and increased risk of disease and death with advancing age (Kirkwood and Holliday, 1997). The manifestation of reactive oxygen species (ROS) and the organism's fight to neutralize its damage result in oxidative stress, a proximate cause of ageing. Metabolically costly activities such as reproduction may favour ROS production (Kramer et al., 2021; Selman et al., 2012). If organisms invest into antioxidants, ROS can be neutralized before critical damage occurs (Münch et al., 2008; Ray et al., 2012; Seehuus et al., 2006).

Social insects (ants, termites, and some bees and wasps) are excellent models to study the evolution of senescence. Obligate reproductive division of labour has led to distinct phenotypes with varying morphologies, behaviours, lifespans, and fecundities. Queens are highly fertile and long-lived, while workers are typically sterile and short-lived (Keller, 1998; Kramer et al., 2016), representing a reversal of the typical trade-off between longevity and fecundity. This may be attributed to the ample resources queens receive from their colonies (Hölldobler and Wilson, 1990; Kramer et al., 2022; Negroni et al., 2019). Although the molecular mechanisms driving caste-specific ageing are not fully resolved, queens are known to invest more into body repair and maintenance, contributing to their extended lifespans, which may last for decades in ants and termites (Lin et al., 2021; Negroni et al., 2019).

Queen lifespan is also influenced by environmental and social factors. In ants, the average life expectancy of a queen is much higher in species with single-queen societies than in polygynous species with several queens per colony (Keller and Genoud, 1997).

Chapter 1

Queen number in the colony is often linked to the colony founding strategy. In monogynous species, queens are well-provisioned and typically found colonies independently (e.g., *Lasius niger*; Janet, 1907). In contrast, queens of polygynous species often mate locally and stay in their natal nest (e.g., *Formica selysi;* Avril et al., 2019; Keller, 1995). Queen loss in monogynous societies ultimately leads to the demise of the colony and impacts worker behaviour and physiology. In polygynous societies, losing one queen has minor consequences as queens can be easily replaced.

Worker reproduction under queenright conditions is rare, likely due to the emission of pheromones by queens indicating their presence and fecundity (Holman, 2018; Oliveira et al., 2020; Bourke, 1988; Foitzik and Herbers, 2001). Worker sterility is found in many invasive species and more generally in species with large colonies (Aron et al., 2001; Dijkstra and Boomsma, 2006). Under queenless conditions, workers of many ants develop their ovaries, fight over reproductive dominance and lay haploid, male-destined eggs (Boomsma, 2009; Heinze et al., 1997). The onset of egg production by queenless workers is typically associated with shifts in their physiology and immunity. These workers exhibit increased oxidative stress resistance, altered brain and fat body gene expression, and frequently have extended lifespans compared to queenright workers (Blacher et al., 2017; Dixon et al., 2014; Hartmann and Heinze, 2003; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021). Worker fecundity is also influenced by age and role within the colony (Bourke, 1988). Younger workers care for brood, while older workers engage in riskier tasks like foraging and defence (Tofilski, 2002; Wilson, 1985, 1971). This age polyethism connects age, behaviour, fecundity, and nest location, with younger inside workers more likely to activate their ovaries under queenless conditions (Blacher et al., 2017; Bourke, 1988; Giraldo and Traniello, 2014; Seistrup et al., 2023).

In contrast to species where workers have functional ovaries, sterile workers cannot reproduce, even in queenless nests. Thus, they should not break the aforementioned longevity/ fecundity trade-off. For instance, queen loss in the supercolonial ant *Lasius neglectus* did not affect the susceptibility to oxidative stress and gene expression in sterile workers (Majoe et al., 2024). In species with functionally sterile workers, workers may still have ovaries and lay nutrient-rich, non-viable trophic

eggs that serve as high-quality food for larvae but cannot develop into offspring. By using these eggs to rear the queen's offspring, workers can increase their indirect fitness but cannot achieve direct fitness benefits. Studying invasive, polygynous species with functionally sterile workers who are unlikely to experience queen loss in nature, may help determine if extended worker lifespans seen in species with reproductive workers also apply to functionally sterile workers with active ovaries that lay trophic eggs.

The dolichoderine ant *Tapinoma magnum* is an ideal system for studying invasive species dynamics. As part of the West and Central Mediterranean *Tapinoma nigerrimum* complex, it has the broadest distribution and invasive potential (Dekoninck et al., 2016; Seifert et al., 2017). In its invasive range, *T. magnum* is highly polygynous and forms supercolonies with thousands of workers and up to 350 queens per nest spot. Most mated young queens remain near their natal colony and are adopted into neighbouring nests (Seifert et al., 2017). Workers display a size polymorphism (Dekoninck et al., 2016; Seifert et al., 2017) and possess well-developed ovaries (pers. observation); however, worker-laid eggs appear to be non-viable trophic eggs, as we did not observe them to develop beyond the egg stage.

Previous studies that investigated the relationship between longevity and fecundity in social insects primarily examined the effects of queen loss in monogynous or facultatively polygynous species, revealing a positive link between worker survival and fecundity, driven by enhanced investment in antioxidants and detoxification enzymes under queenless conditions (Majoe et al., 2021; Negroni et al., 2021b). In this study, we extended this framework by examining the effects of queen loss, monogyny, and polygyny on worker survival, ovarian development, oxidative stress resistance, and fat body gene expression (linked to energy storage and reproduction; Arrese and Soulages, 2010; Cervoni et al., 2017) in a strictly polygynous species. Additionally, we explored whether queen loss or queen number elicits similar physiological and molecular responses in workers (Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021b). Since age-related division of labour is often linked to physiological changes in ovary development and oxidative stress resistance (Foitzik et al., 2002; Giraldo and Traniello, 2014; Hartmann and Heinze, 2003; Quque et al., 2023), we hypothesized that worker location would correlate with age, ovary development, oxidative stress resistance, and

fat body gene expression (Koto et al., 2023; Kramer et al., 2021; Majoe et al., 2021; Seistrup et al., 2023). We expected inside workers to exhibit stronger physiological responses to queen number than outside workers, as they interact more closely with the queen. Additionally, we predicted that inside workers would demonstrate greater resistances to oxidative stress, consistent with findings in other species (Kennedy et al., 2021; Kramer et al., 2021; Majoe et al., 2021).

Within a complementary experiment, we explored how queen age influences fecundity and gene expression. Age-related changes in queens influence reproduction and molecular pathways (Negroni et al., 2019; Von Wyschetzki et al., 2015), while queen lifespan is often tied to social structure (Hölldobler and Wilson, 1977; Keller and Genoud, 1997; Kramer et al., 2022). Queens of the polygynous form of the red imported fire ant, *Solenopsis invicta*, are estimated to live between one and three years (Goodisman and Ross, 1999), while queens of the similarly invasive dolichoderine ant, *Linepithema humile*, typically have lifespans of approximately one year (Reuter et al., 2001). Although the exact lifespan of *Tapinoma magnum* queens is unknown, our laboratory observations show that dealated queens can live well over one year. Based on these observations and the estimated lifespans of comparable invasive and polygynous species, we hypothesized that *T. magnum* queens may have average lifespans in the range of one to three years (Goodisman and Ross, 1999; Keller and Genoud, 1997; Reuter et al., 2001). In short-lived *Cardiocondyla obscurior* queens, fecundity remains consistent until the end of life, whereas long-lived *Temnothorax rugatulus* queens transition from prioritizing immune function to antioxidant production as they age (Jaimes-Niño et al., 2022; Negroni et al., 2019). Unlike long-lived queens of monogynous ants that can survive for decades, shorter-lived queens, such as those of *T. magnum*, may also exhibit divergent ageing trajectories. By analysing gene expression in the brain and fat body— key tissues for hormone production and protein synthesis (Corona et al., 2007; Min and Benzer, 1997)— we sought to understand how ageing impacts queen physiology. This approach allowed us to compare age-related transcriptional changes in queens and workers, offering a comprehensive perspective on the links between age, reproduction, and molecular changes in *T. magnum*. Together, these experiments illuminate how social structure and ageing shape the physiological and molecular underpinnings of colony dynamics.

Material and Methods

Study site, collection, and laboratory maintenance

We collected ants from a supercolony of *Tapinoma magnum* on a strip of fallow land, formerly a plant nursery, in Ingelheim am Rhein, Germany (49°58'39.8"N, 8°03'17.3"E) in June and July 2020, and in June 2021. In 2020, 13 dealate queens, a few thousand workers and brood were collected. In 2021, we collected 13 alate queens, over 30 males, 50 dealate queens, and a few thousand workers and brood from multiple nest chambers, each typically housing two to four queens. Colonies were maintained in a 25°C climate chamber at the Johannes Gutenberg University Mainz. The 13 dealate queens and workers from 2020 and the 50 dealate queens and workers from 2021 were housed in separate boxes (78 cm x 56 cm x 18 cm), coated with Fluon® (Whitford GmbH, Diez, Germany) to prevent escapes. Each box contained three artificial nests, each constructed from two glued Petri dishes connected by a 1 cm hole plugged with cotton. The lower Petri dish (7.5 cm diameter, 4 cm high) served as a water reservoir, the upper Petri dish (9.5 cm diameter, 1.2 cm high) functioned as nest entrance and was covered with a loose lid containing small holes. Each nest was covered by a plastic flowerpot (13 cm diameter, 12 cm high) to create a dark environment. Colonies were fed twice weekly with a mix of honey, eggs, and vitamins, and once weekly with crickets. Queens collected in 2020 were maintained under these conditions for at least 16 months, while alate queens from 2021 were housed in a separate box (78 cm x 56 cm x 18 cm) with males to ensure fertilization. After copulation, the young queens shed their wings and became reproductive.

Experimental set-up

To investigate the effect of queen number on worker survival, ovarian development, and gene expression, we divided the source colony collected in 2021 into 10 replicate sets, each containing three sub-colonies housed in separate boxes (40 cm x 33.5 cm x 17 cm) with one nest covered by a plastic flowerpot (as described above). Each sub-colony was randomly assigned to one of three treatments: “queenless” (no queen), “monogynous” (one queen), and “polygynous” (two queens). The polygynous treatment simulated the

natural queen-worker interactions of nests with two to four queens per chamber. Inside workers were collected near or on the brood pile. Outside workers were collected in the foraging arena outside the nest. Each sub-colony consisted of 176 inside and 176 outside workers, evenly split between large and small individuals (1:1 ratio; Fig. 1A), along with similar amounts of eggs and larvae. Workers were visually categorized as large or small based on a single observation. We confirmed that large workers had wider heads– a typical proxy for ant body size– than small workers (Welch's t-test: N = 389, t = 19.037, df = 368.71, $p < 0.0001$; see Supplement for details). Workers were then randomly assigned to each sub-colony. Each replicate set received a unique ID, each referred to as "colony ID". Since it was not feasible to initiate all replicate sets simultaneously, the experiment was conducted in six different phases, each referred to as a "cohort". The six cohorts started four weeks apart with four cohorts containing two replicate sets each and two cohorts with one replicate set each. Ants were maintained in their treatments for 58 days, with worker mortality documented twice per week by counting and removing dead workers. Pupae were removed before emergence. Based on similar studies (Choppin et al., 2021; Kohlmeier et al., 2017; Majoe et al., 2021) and pilot observations of the laboratory set-up, we chose a 58-day duration for this experiment. We focused on large workers to compare ovarian development and gene expression between inside and outside workers in response to queen number, avoiding additional complexity resulting from including different worker morphs that did not address our research questions. After 58 days, large inside and outside workers were randomly chosen from each treatment (two samples per worker location, N = 12) within one replicate set per cohort (6 replicate sets in total) and frozen at -80°C. Ovary and fat body dissections were performed simultaneously, with dissections and RNA extractions (see Supplement for details) conducted randomly (Fig. 1D).

To further explore the role of age in reproduction and gene expression, we conducted a complementary experiment on queens. Queens collected in 2020 were at least 16 months old and likely older, as they were already wingless at collection, and were classified as "old" in our transcriptomic analysis. Alate queens collected in 2021 were not older than three months when sampled and classified as "young". After the young queens became reproductive, we set up 12 sub-colonies, each with one young

and one old queen (24 queens total) and 200 large and small workers from inside and outside the nests from both collection years. The queens were marked with thin wires of different colours to denote their age. The queens were maintained in these standardized sub-colonies for 14 days to ensure a controlled environment for all queens prior to sampling. Thereafter, the queens were frozen at -80°C for later ovary, brain, and fat body dissection for fecundity and gene expression analyses.

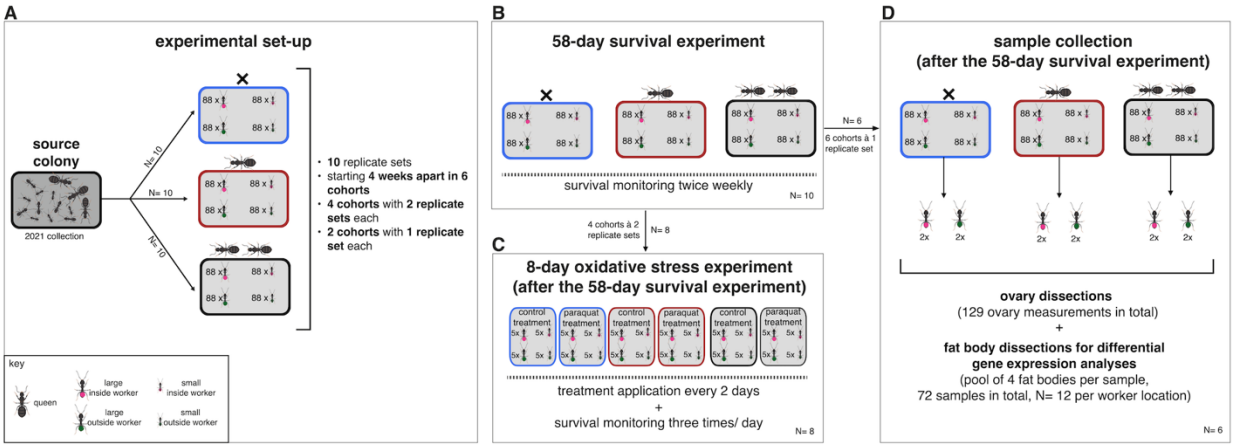

Figure 1: Experimental set-up.

A) The source colony was divided into 10 replicate sets, each containing three sub-colonies with no queen (queenless treatment, blue box, N = 10), one queen (monogynous treatment, red box, N = 10) and two queens (polygynous treatment, grey box, N = 10). Each sub-colony consisted of 352 workers comprising of similar numbers of large/small inside and outside workers. The 10 replicate sets started at six time-points (cohorts) four weeks apart (four cohorts à two replicate sets and two cohorts à one replicate set). **B)** Worker mortality was documented twice per week for 58 days. **C)** For the oxidative stress experiment, 10 large and 10 small inside workers, as well as 10 large and 10 small outside workers from each treatment (N = 8) were either subjected to an oxidative stress treatment (Paraquat dichloride solution) or control treatment (Millipore water) for eight days. Treatments were repeated every two days and survival was monitored three times daily. **D)** For the ovary dissections and the differential expression analyses, large inside (pink, N = 12) and outside (green, N = 12) workers from the three treatments were used. We used two samples per worker location across six replicate sets from six cohorts (N = 6). Each fat body sample (72 samples) was a pool of four individual fat bodies used for differential expression analysis. Ovary and fat body dissections were conducted simultaneously. We measured 129 worker ovaries (out of 144 dissected workers).

Oxidative stress experiment

After the 58-day survival experiment, we randomly selected 10 large and 10 small inside workers, as well as 10 large and 10 small outside workers, from each treatment (8 replicate sets; four cohorts). The workers were colour-marked with paint markers (Edding® 750) and kept in small plastic boxes (10 cm x 10 cm x 5 cm). We randomly assigned five large and five small inside workers, five large and five small outside workers to either the control or the oxidative stress treatment (Fig. 1C). The next day, workers assigned to the oxidative stress treatment (480 workers per treatment) were subjected to a 0.46 M solution of Paraquat dichloride ($CH_3(C_5H_4N)_2CH_3 \cdot 2Cl$) dissolved in Millipore water. Paraquat is an herbicide inducing oxidative stress through superoxide formation (Cousin et al., 2013). We applied paraquat solution twice to the dorsal surface of each worker's head using a size 1 Vernissage™ paintbrush. Head width correlates with body size in ants and determined the approximate amount applied. This method was adapted from Majoe et al. (2021), who successfully used similar concentrations and application protocols across ant species of varying sizes. After application, each ant was isolated for two hours in a 1.5 ml Eppendorf tube to avoid the transfer of the solution to other ants via trophallaxis (mouth-to-mouth fluid exchange), and to facilitate self-grooming and ingestion, as Paraquat requires ingestion to manifest its effects. Workers of the control treatment were treated similarly but pure Millipore water instead of the paraquat solution was applied. These treatments were repeated every two days over a period of eight days and worker survival was monitored three times per day (Majoe et al., 2021).

Ovary dissections

To assess the impact of queen number and worker location on ovarian development, we dissected 144 large inside and outside workers under a stereomicroscope (Leica S9i, Microsystems CMS GmbH, Wetzlar, Germany) with magnification ranging from 1.25x to 5.0x. Images (2592 x 1944 px) of the ovaries were captured using Leica LAS software (v.4.12.0). We measured the ovariole length of 129 workers using ImageJ2 Fiji software (v.2.9.0). The mean of the two longest ovarioles was calculated (typically four ovarioles per worker, Supplementary Figure S1) and the total number of developing oocytes was

counted (Fiji software). To assess the impact of age on queen fecundity, we dissected 24 queens and measured mean ovariole length as described for the workers. The ovaries from one young and one old queen were lost during dissections, resulting in 22 measured queen ovaries. We also counted the number of oocytes (Leica LAS software). All queens had sperm-filled spermathecae.

Statistical analyses

The *coxme* package (v.2.2-18.1; Therneau, 2024) in RStudio (R v.4.2.3; R Core Team, 2024) was used to build two Cox-regression mixed-effects models. One model was created to analyse the 58-day survival experiment and one model for the oxidative stress experiment. The variable "queen number" (queenless/ monogynous/ polygynous) was included as fixed factor, and "experimental box" (a combination of "colony ID" and "cohort") as random factor to account for variability associated with both factors. To compare whether worker survival depended on queen number, pairwise comparisons were performed for: queenless vs. monogynous; queenless vs. polygynous; and monogynous vs. polygynous (Holm-Bonferroni adjusted p-values).

For the oxidative stress experiment, we first constructed a Cox-regression mixed-effects model to assess how treatment (oxidative stress/ control) influenced worker survival, accounting for potential interactions with queen number, worker location (inside/ outside), and worker size (large/ small). Fixed factors included "treatment", "queen number", "worker location", and "worker size", and their interactions with treatment. "Experimental box" was included as random factor. To investigate worker survival in each treatment group (oxidative stress/ control) separately, we constructed additional Cox-regression mixed-effects models for each treatment. These models included "queen number", "worker location", "worker size", and their interactions as fixed factors, allowing us to assess how worker location and size influenced survival under varying queen numbers in each treatment. "Experimental box" was included as random factor. Non-significant interactions were removed and the package *emmeans* v.1.10.2 (Lenth, 2024) was used to perform pairwise comparisons between groups. Hypothesis testing was performed using the 'Anova' function from the package *car*

(v.3.1-2; Fox et al., 2024). The package *ggplot2* (v.3.4.2; Wickham, 2016) was used to plot the Kaplan-Meier survival curves. All model fits (58-day survival and oxidative stress experiment) were assessed by visualizing the distribution of fitted values using histograms, Quantile-Quantile plots (Q-Q-plots; R function qqnorm), and overlaid Q-Q lines (R function qqline) to identify deviations from expected distributions and confirm model appropriateness.

We modelled the mean ovariole length of large inside and outside workers using a linear mixed-effects model from the package *lme4* (v.1.1-33; Bates et al., 2015) and tested the effect of the explanatory variables with the 'Anova' function (*car* package). The number of oocytes in development was modelled using a generalized linear mixed-effects model (*lme4* package) with a 'Poisson' distribution and tested with the 'Anova' function. All models included "queen number", "worker location", and their interaction as fixed effects, with "sub-colony ID" as random factor. Each sub-colony ID inherited colony ID and cohort information, accounting for variability associated with both factors. The mean ovariole length and the number of oocytes of queens were analysed using a linear mixed-effects model with the *lme4* package. "Mean ovariole length" and "oocyte number", respectively, were included as the response variable, "queen age" (young/ old) as fixed factor and "colony ID" as a random factor. Hypothesis testing was performed with the 'Anova' function. We performed diagnostic checks on the residuals for all models (for workers and queens), including Q-Q plots (R function qqnorm) of the residuals and overlaid Q-Q lines (R function qqline) to ensure model appropriateness. For all analyses, we used a significance threshold of $p < 0.05$.

Differential expression analysis

For the differential expression analysis, we used 72 worker samples (fat body) and 23 queen samples (brain and fat body). Detailed information on RNA extraction and sequencing can be found in the Supplement. Raw RNAseq read quality was assessed, and filtering was performed to remove adaptors using Fastp (v.0.2; Chen et al., 2018). Post-filtered sequence quality was assessed using FastQC (v.0.11.8; Andrews, 2010) with the data being of high quality to perform differential expression analyses. Since

there is no reference genome assembly available for *Tapinoma magnum,* we generated a *de novo* transcriptome assembly using Trinity (v.2.13.2; Grabherr et al., 2011) with the SuperTranscripts option (Davidson et al., 2017).To generate the transcriptome assembly, we used a combination of the filtered queen and worker reads. To quantify transcript abundance, RSEM (v.1.3.3; Li and Dewey, 2011) was used, resulting in an overall alignment rate of 79.4% ± 1.5 (mean ± sd) for the worker dataset and 76.7% ± 2.5 (mean ± sd) for the queen dataset. One old queen brain sample was removed from further analyses due to a low mapping rate of 47%, leaving 23 queen samples. Differential gene expression analysis was conducted in RStudio (R v.4.2.3). For each dataset, transcript abundances were introduced into estimated count matrices with the package *tximport* (v.1.26.1; Soneson et al., 2016), serving as input for differential gene expression analyses using the *DESeq2* package (v.1.38.3; Love et al., 2014). Subsequent transcriptome-based analyses were conducted separately for the worker and queen dataset.

For the worker dataset, contigs with zero reads in at least 11 of 12 replicates were removed from the count matrix, resulting in 73,277 contigs retained in the differential expression analysis. We first examined whether the impact of queen number on gene expression differed between inside and outside workers as we expected inside workers to react more strongly to varying queen numbers than outside workers due to their closer interactions with the queen. To identify genes whose expression was affected by the interaction between queen number and worker location, we compared the following models:

Full model: ~colony ID+ queen number+ worker location+ queen number: worker location
Reduced model: ~colony ID+ queen number+ worker location

Second, to test the main effect of queen number, we compared these models:

Full model: ~colony ID+ worker location+ queen number
Reduced model: ~colony ID+ worker location

Third, to examine the main effect of worker location on gene expression, we compared the following models:

Full model: ~colony ID+ queen number+ worker location
Reduced model: ~colony ID+ queen number

To identify groups of differentially expressed genes with similar expression patterns, we conducted a cluster-based analysis with the R package *DEGreport* (v.1.34.0; Pantano, 2017).

For the queen-based analysis, separate analyses were performed for each tissue. Only contigs with more than zero reads in at least five out of six replicates per tissue were kept, resulting in 55,349 contigs for the differential expression analysis. The following model comparison was performed to address our research question:

Full model: ~age
Reduced model: ~1

All model comparisons were conducted using Likelihood Ratio Tests (LRT) as implemented in *DESeq2* with Benjamini-Hochberg adjusted p-values of 0.05 used as a threshold to determine whether a gene was significantly differentially expressed.
To explore the relationship between age and gene expression in workers and queens, and to assess if worker location can serve as a proxy for age, we compared differentially expressed genes that were upregulated in inside or outside workers with those upregulated in young or old queens using Venny (v.2.1; Oliveros, 2007). Fisher's exact tests (significance threshold $p < 0.05$) examined the significance of overlaps between inside workers vs. young queens, outside workers vs. old queens, inside workers vs. old queens, and outside workers vs. young queens, using a total pool of 52,093 contigs (common number of contigs in workers and queens).

Gene annotation and functional enrichment analyses

To annotate the Trinity-assembled transcriptome, we conducted a BlastX homology search against the non-redundant invertebrate protein database (Altschul et al., 1990; downloaded July 2023). Nucleotide sequences were translated into predicted amino-acid sequences using TransDecoder (v.5.7.0-Perl-5.30.0;Haas and Papanicolaou, 2017), which were then used as input for InterProScan (v.5.54-87.0; Blum et al., 2021) for the identification of conserved functional domains and assignment of associated Gene Ontology (GO) terms to our *de novo* transcriptome assembly. We additionally used OrthoFinder (v.2.5.4; Emms and Kelly, 2019) to examine potential homologues between the closely related dolichoderine ant *Linepithema humile* (Ensembl Metazoa Biomart) and *Tapinoma magnum*, resulting in the identification of 5,859 potential homologues, of which 63% could be assigned additional GO terms based on homology to *L. humile*. We combined both sets of GO terms to create a GO term database, which was then used in our GO term enrichment analysis. To identify which terms attributed molecular functions functionally enriched within workers and queens, we performed individual GO term enrichment analyses based on the differentially expressed contigs using Fisher's exact tests (significance threshold $p < 0.05$) implemented by the package *TopGo* (v.2.50.0; Alexa and Rahnenführer, 2024) using the weight01 algorithm. The scripts used for these analyses were modified versions of those developed by Colgan et al., (2019).

Results

Queen number influenced worker survival, while worker location was associated with ovarian development

To investigate the effect of queen number on worker survival, we monitored worker survival in queenless, monogynous, and polygynous colonies over 58 days. We found that queen number influenced worker survival, as workers from monogynous sub-colonies survived best, followed by workers of polygynous colonies, and then workers of queenless colonies (queen number 0 vs. 1: $\chi^2 = 155$, $p < 0.001$; 0 vs. 2: $\chi^2 = 41.6$, $p < 0.001$; 1 vs. 2: $\chi^2 = 51.7$, $p < 0.001$; Fig. 2A). We further examined the influence of queen number and worker location on ovarian development by measuring ovariole length and counting

the number of developing oocytes in large inside and outside workers from each queen treatment. The workers had a mean ovariole length of 1.86 mm (± 0.4 mm sd), which was independent of queen number and worker location ($LMER_{queen\ number}$: $\chi^2 = 1.17$, $p = 0.56$; $LMER_{worker\ location}$: $\chi^2 = 2.75$, $p = 0.10$; Supplementary Figure S2). In contrast, inside workers had more developing oocytes than outside workers (GLMER: $\chi^2 = 77.11$, $p < 0.0001$; Fig. 2B). Moreover, workers of monogynous sub-colonies had more oocytes than workers of polygynous sub-colonies (GLMER: $\chi^2 = 4.32$, $p = 0.037$; Fig. 2B), with an interaction between queen number and worker location (GLMER: $\chi^2 = 6.65$, $p = 0.009$).

Worker location but not queen number influenced worker resistance to oxidative stress

To test whether queen number, worker location, and worker size influenced the resistance of workers to oxidative stress, we subjected large and small inside and outside workers from each queen treatment to either a paraquat-induced oxidative stress treatment or the control treatment (Millipore water). The oxidative stress treatment reduced worker survival compared to the control treatment ($\chi^2 = 306.96$, $p < 0.0001$), while queen number did not influence worker survival in both treatment groups ($\chi^2 = 1.53$, $p = 0.466$). We found significant interactions between the treatment groups and the fixed factors queen number ($\chi^2 = 6.986$, $p = 0.03$), worker location ($\chi^2 = 8.769$, $p = 0.003$), and worker size ($\chi^2 = 6.261$, $p = 0.012$) and subsequently investigated worker survival within each treatment group separately. In the control treatment, we found weak evidence that queen number influences worker survival ($\chi^2 = 4.79$, $p = 0.091$). Closer examination revealed that this trend followed a similar pattern as to what we observed after our 58-day survival experiment (queen number 0 vs. 1: $z = 1.808$, $p = 0.167$; 0 vs. 2: $z = -0.242$, $p = 0.968$; 1 vs. 2: $z = -2.043$, $p = 0.102$). Inside workers survived better than outside workers in both, the control, and the oxidative stress treatment (control: $\chi^2 = 37.78$, $p < 0.0001$; oxidative stress: $\chi^2 = 26.81$, $p < 0.0001$, Fig. 2C/D), while worker survival in the control was lower compared to the 58 - day survival experiment (Fig. 2A). Additionally, large workers survived better under oxidative stress than small workers ($\chi^2 = 46.11$, $p < 0.001$), while we found very weak

evidence that worker size influenced survival in the control treatment ($\chi^2 = 2.69$, $p = 0.10$; Supplementary Figure S3).

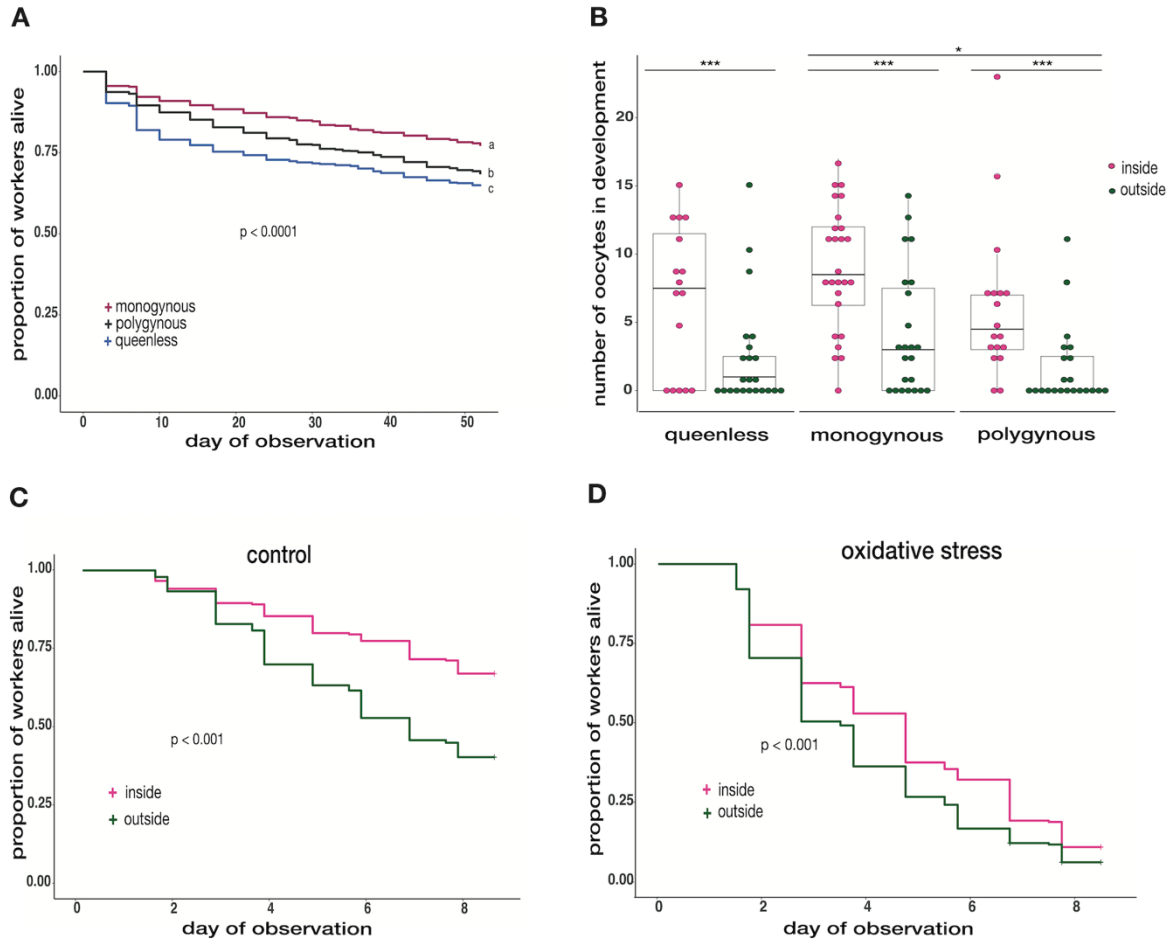

Figure 2: Worker survival, ovarian development, and oxidative stress resistance dependent to queen number and worker location.

A) Worker survival over 58 days under queenless, monogynous and polygynous conditions. Workers of monogynous (red line) colonies survived best, followed by workers of polygynous (grey line) colonies and workers of queenless (blue line) colonies (Queen number 0 vs. 1: $\chi^2 = 155$, $p < 0.001$; 0 vs. 2: $\chi^2 = 41.6$, $p < 0.001$; 1 vs. 2: $\chi^2 = -51.7$, $p < 0.001$). **B)** The number of oocytes dependent on queen number and worker location. Inside (pink) workers had more oocytes in development than outside (green) workers (GLMER: $\chi^2 = 77.11$, $p < 0.0001$). Workers of monogynous colonies developed more oocytes than workers of polygynous colonies (GLMER: $\chi^2 = 4.32$, $p = 0.037$), with an interaction between queen number and worker location (GLMER: $\chi^2 = 6.65$, $p = 0.009$). **C)** Worker survival in the control treatment (oxidative stress experiment). Inside (pink line) workers had a higher survival probability than outside (green line) workers ($\chi^2 = 37.78$, $p < 0.0001$). **D)** Worker survival in the oxidative stress treatment. Inside workers showed a higher resistance to oxidative stress than outside workers ($\chi^2 = 26.81$, $p < 0.0001$).

Worker location rather than queen number influenced fat body gene expression in workers

To investigate the effect of queen number and worker location on fat body gene expression (72 samples), we analysed gene expression differences between inside and outside workers across treatments (queenless, monogynous, polygynous). We found no evidence that queen number affected fat body gene expression (0 differentially expressed genes, all BH-adjusted p-value > 1), while worker location was linked to strong transcriptomic differences. Between inside and outside workers, we identified 4,305 significantly differentially expressed genes (DEGs; BH-adjusted $p < 0.05$). Of these, 2,188 showed an elevated expression in inside workers, while 2,117 DEGs were elevated in outside workers. We identified 108 DEGs, whose expression was affected by an interaction between worker location (inside/ outside) and queen number (queenless/ monogynous/ polygynous), of which, 102 were grouped into three co-expression clusters (Fig. 3A). The first cluster consisted of 31 genes whose expression in inside workers decreased with increasing queen number. Within outside workers the expression was lower in queenless colonies and was similarly increased in monogynous and polygynous colonies. Cluster 2 consisted of 36 genes where expression was highest in inside workers sampled from queenless colonies, while, similar to cluster 1, the expression was decreasing with queen number. In outside workers, the difference in expression between queenless and monogynous colonies was even stronger compared to cluster 1. The expression in monogynous and polygynous colonies was similarly high. The blast homology search identified two genes in cluster 1 (*serine protease inhibitor ¾-like isoform X3,* LRT: BH-adjusted p= 0.048; *group XIIA secretory phospholipase A2*, LRT: BH - adjusted p = 0.049) and two genes in cluster 2 (*ribosome biogenesis protein WDR12 homolog,* LRT: BH - adjusted p = 0.049; *cell division cycle protein 123 homolog isoform X1,* LRT: BH - adjusted p = 0.037). Finally, cluster 3 contained 35 genes with expression patterns opposite to the first two clusters (Fig. 3A). In inside workers, expression profiles increased with queen number, while outside workers showed the highest expression in queenless colonies, which was decreasing in monogynous and polygynous colonies. Here, we found nine genes related to immunity and detoxification (*e.g.*, *xanthine dehydrogenase 1-like*, LRT: BH - adjusted p = 0.037; *glucose dehydrogenase*, LRT:

BH - adjusted p = 0.047; Kim et al., 2001; Lee et al., 2005), three genes involved in the Ras signalling pathway (*e.g.*, *ras-like GTP-binding protein Rho1 isoform X2*, LRT: BH - adjusted p = 0.013), and *juvenile hormone epoxide hydrolase 1-like* (LRT: BH - adjusted p = 0.047).

Genes with higher expression in inside workers were enriched for 10 Gene Ontology (GO) terms (Fig. 3B, Supplementary Table S1), with the most significantly enriched terms being *oxidoreductase activity* (Fisher's exact p < 0.0001), *iron ion binding* (Fisher's exact p < 0.0001), and *monooxygenase activity* (Fisher's exact p < 0.0001). In contrast, 19 enriched GO terms were identified for DEGs upregulated in outside workers. Next to terms also represented in inside workers, we identified terms related to lipid metabolic processes (e.g., *lipase activity,* Fisher's exact p = 0.036) and proteolysis (e.g., *metallocarboxypeptidase activity*, Fisher's exact p < 0.001), drawing a more diverse expression profile in outside workers (Fig. 3C; Supplementary Table S2).

Within the genes upregulated in inside workers, our blast homology search identified the antioxidants *superoxide dismutase [Cu-Zn]-like* (LRT: BH - adjusted p = 3.83E-11, Fig. 3D), *transferrin* (LRT: BH - adjusted p = 0.003), as well as *protein toll* (LRT: BH - adjusted p = 6.64E-05, Fig. 3E). Moreover, we found *vitellogenin-1* (LRT: BH - adjusted p = 0.045), *vitellogenin-1-like* (LRT: BH - adjusted p = 0.012), and *vitellogenin-2-like* (LRT: BH - adjusted p = 0.029, Fig. 3F; all *conventional vitellogenins*, *C-Vg;* Kohlmeier et al., 2018). Within the genes upregulated in outside workers, we found higher expression of *dual oxidase isoform X2* (LRT: BH - adjusted p = 0.035, Fig. 3G), 22 zinc finger proteins (*e.g., zinc finger protein 846-like*, LRT: BH - adjusted p = 6.74E-07, Fig. 4H) as well as 24 DEGs involved in lipid metabolism (*e.g.*, *fatty acid synthase*, LRT: BH - adjusted p = 0.001, Fig. 3I).

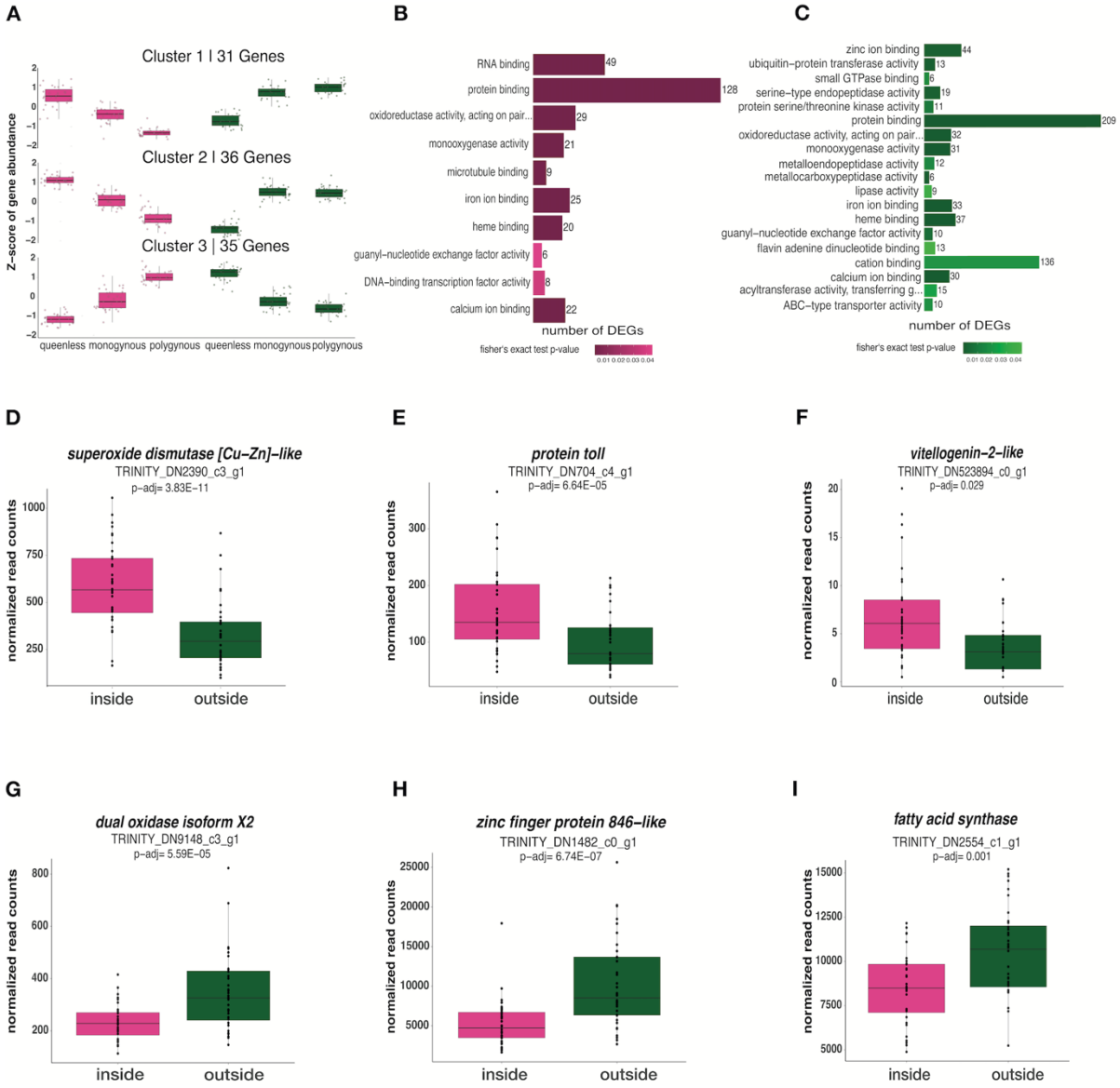

Figure 3: The influence of queen number and worker location on fat body gene expression.

A) 102 out of the 108 DEGs from the interaction term (queen number : worker location) were grouped into three clusters based on the expression profiles within each worker caste (pink = inside workers; green = outside workers) and social environment**. B)** The 10 most enriched GO terms (molecular function) in the list of genes upregulated in inside workers and **C)** the 19 most enriched GO terms (molecular function) in the list of genes upregulated in outside workers. The size of each bar correlates with the number of DEGs associated with each term, while the colour correlates with the p-value (the darker the colour, the lower the p-value). **D)-I)** Selected DEGs with notable function within inside workers and outside workers.

Queen age elicited no changes in fecundity but minor transcriptional shifts in fat body gene expression

To investigate the effect of age on queen fecundity and gene expression, we dissected the ovaries (22 queen ovaries) and analysed brain and fat body gene expression of young and old *T. magnum* queens (23 samples in total). Age did not influence fecundity as young queens had similarly long ovarioles and as many oocytes in development as old queens ($LMER_{ovariole\ length}$: $\chi^2 = 0.02$, $p = 0.811$; $LMER_{oocyte\ number}$: $\chi^2 = 0.04$, $p = 0.836$; Supplementary Figure S4).

No age-related transcriptomic changes were detected in the brain (11 samples). However, 126 DEGs were identified in the fat bodies between young and old queens (12 samples). Of these, 75 DEGs were upregulated in old queens, while 51 DEGs were upregulated in young queens. In old queens, we found an overexpression of *chymotrypsin-2-like* (LRT: BH - adjusted p = 0.0009, Fig. 4A), *vitellogenin-1-like* (LRT: BH - adjusted p = 0.005), and *vitellogenin-2-like* (LRT: BH - adjusted p = 0.0001; Fig. 4B; *C-Vg;* Kohlmeier et al., 2018). Young queens showed increased expression of *hexamerin - like* (LRT: BH - adjusted p = 0.0013, Fig. 4C) and *acyl-CoA Delta(11) desaturase-like* (LRT: BH - adjusted p= 0.039, Fig. 4D*)*. We could not identify significantly enriched GO terms within the DEGs between young and old queens.

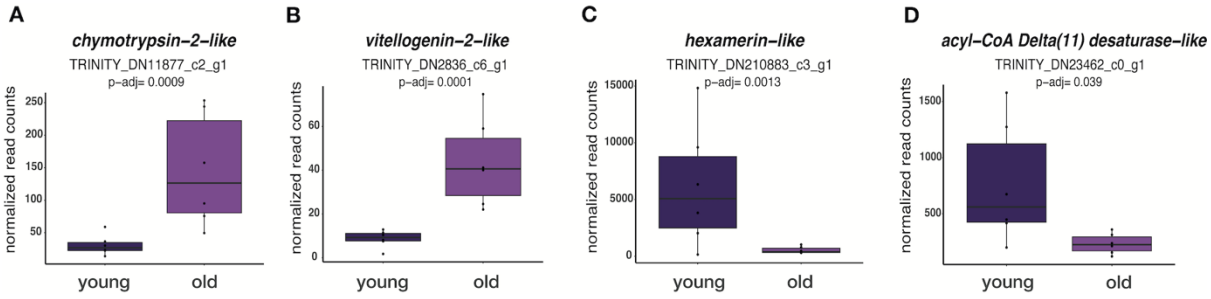

Figure 4: Candidate DEGs of interest differing in expression in the fat body between young and old queens.

A) Normalized read counts of *chymotrypsin-2-like* and **B)** *vitellogenin-2-like* which are significantly overexpressed in old queens. **C)** Normalized read counts of *hexamerin-like* and **D)** *acyl-Coa Delta(11) desaturase-like* which are overexpressed in young queens.

To explore the link between age and gene expression, we compared DEGs that were upregulated in young and old queens with those upregulated in inside and outside

workers. We found that 20 DEGs were upregulated in inside workers and young queens, and 15 DEGs were upregulated in outside workers and old queens. These overlaps were larger than what could be expected by chance (Fisher's exact test $p < 0.0001$; Fisher's exact test $p < 0.0001$, respectively). Hexamerin storage proteins were enriched in inside workers and young queens. We found four genes to be upregulated in both inside workers and old queens, and five in outside workers and young queens, and we cannot reject the hypothesis that these overlaps may be explained by chance (Fisher's exact test $p = 0.558$, Fisher's exact test $p = 0.055$, respectively; see Supplement for details).

Discussion

Physiological and transcriptional effects of queen number and worker location

We investigated whether queen number and worker location affected worker survival, ovarian development, oxidative stress resistance, as well as fat body gene expression in the invasive ant *Tapinoma magnum*. Our two-month survival experiment revealed that workers of monogynous colonies survived best, followed by workers of polygynous colonies, with the lowest survival in workers of queenless colonies. Workers of monogynous colonies had more oocytes in development compared to those from polygynous colonies, while inside workers had more oocytes than outside workers. Oxidative stress resistance and fat body gene expression were not directly influenced by queen number whereas worker location was strongly linked to both.

Our results differ from previous studies on other ant species, such as *Temnothorax longispinosus* (Kohlmeier et al., 2017), *Acromyrmex echinatior*, *Atta colombica*, and *Temnothorax rugatulus* (Majoe et al., 2021). Specifically, *T. magnum* workers exhibited the highest mortality in queenless colonies, contrasting with the increased lifespan observed in queenless workers of these species. While worker reproduction has been observed in the invasive ant *Anoplolepis gracilipes* (Lee et al., 2017), it remains unclear whether worker reproduction increases following queen loss or how it impacts worker lifespan. More recently, Hamidi et al., (2023) demonstrated that workers of the highly

polygynous ant *Crematogaster pygmaea* can produce males in the absence of queens. However, the potential link between this reproductive investment and worker lifespan remains uncertain. The effects of queen loss in strictly polygynous species with functionally sterile workers remain understudied. Our results indicate that queen loss decreases worker survival, without influencing ovarian development. While the complete loss of all queens in a supercolony is highly unlikely, queenlessness in a single nest chamber is more probable and can cause stress to the workers. Consequently, increased metabolic rates and ROS production may increase mortality (Pearl, 1928). Although *T. magnum* is highly polygynous, our results demonstrate that workers respond to differences in queen number, indicating that workers are sensitive not only to queen presence but also to the number of queens in their local nest.

The higher survival in monogynous colonies might be linked to the increased oocyte development and presumably increased trophic egg-laying in workers kept in monogynous compared to polygynous conditions. Trophic eggs provide essential nutrients, especially proteins, for growth and reproduction in larvae and queens (Gobin et al., 1998; Gobin and Ito, 2000). In monogynous colonies, egg production hinges on the sole queen. Queen fecundity and nutritional care provided to the queens are likely correlated (Hannonen et al., 2002; Trettin et al., 2011; Chen and Vinson, 2000), while queens may receive less food under polygynous conditions compared to monogynous conditions (Keller, 1988). Increased trophic egg-laying might be linked to a higher nutritional care provided to the single queen. Workers might have increased their own lifespan to increase the fitness and survival of the single queen, ultimately ensuring colony survival. Alternatively, workers may increase their lifespans by shifting to intranidal tasks to support the colony's needs (Calabi and Traniello, 1989; Robinson et al., 2009; Schultner et al., 2017).

Queen number did not directly affect worker survival under oxidative stress, though very weak evidence suggested workers from monogynous conditions survived better in the control treatment. Worker survival in the control treatment was lower than in the 58-day experiment, likely because the oxidative stress experiment occurred after the two-month experiment, when workers had a shorter residual lifespan. Differences in oxidative stress resistance were primarily linked to worker location, supporting our

hypothesis that younger inside workers invest more into body repair. Since outside workers are likely older, this investment may not be worthwhile given their shorter residual lifespan (Kohlmeier et al., 2017; Majoe et al., 2021). Alternatively, the pronounced impact of paraquat exposure may have masked any potential effects associated with queen number.

Our transcriptomic analysis revealed that inside and outside workers responded with divergent transcriptional shifts to queen number. Genes of interest in the first cluster were *serine protease inhibitor ¾-like isoform X3* and *group XIIA secretory phospholipase A2*. Serine protease inhibitors are involved in immune responses (Kanost, 1999; Kanost and Clarke, 2005), while the inhibition of phospholipases A2 (PLA_2) impairs egg-laying behaviour, metabolism, and immunity in insects (Stanley and Kim, 2019). In queenless colonies, these genes were among those expressed the most by inside workers, and less by outside workers. In cluster 2, genes of interest were related to detoxification (*ribosome biogenesis protein WDR12 homolog*) and the cell division cycle (*cell division cycle protein 123 homolog isoform X1*). Cell cycle division proteins are involved in various biological processes and are often increased after injuries (Pal and Raj, 2022). Thus, inside workers may exhibit a more pronounced response to queenlessness, potentially through the activation of stress- and immunity-related genes. Older outside workers likely faced increased mortality regardless of queen number. This supports our hypothesis that worker responses to queen number vary depending on their location within the nest and possibly their age. In cluster 3, we found more genes related to immunity and detoxification as well as Ras proteins (e*.g., ras-like GTP-binding protein Rho1 isoform X2*), and *Juvenile hormone epoxide hydrolase 1-like (JHEH)*, upregulated in inside workers within monogynous and polygynous colonies compared to queenless colonies, while outside workers overexpressed these genes under queenless conditions. Ras proteins play a central role in cell cycle regulation and tissue repair (Boonstra et al., 1995; Stacey, 2003). *JHEH* plays an important role in the degradation pathways of juvenile hormone (JH). In adult females of *Drosophila melanogaster,* JH is required for oogenesis and reproductive maturation (Dubrovsky et al., 2002). Moreover, gene expression varied less in outside workers within monogynous and polygynous colonies in all three clusters.

Worker location was associated with strong changes in gene expression, likely further explaining the higher resistance to oxidative stress in inside workers. Inside workers invested more into antioxidant genes (*superoxide dismutase [Cu-Zn]-like* and *transferrin*), accompanied by an overexpression of *vitellogenin-2-like* (*C*-Vg; Kohlmeier et al., 2018) and *protein toll-like*. Superoxide dismutase and transferrin are known to eliminate ROS while they are also linked to the individual's reproductive potential (Nojima et al., 2015; Tasaki et al., 2018). Vg proteins are synthetized in the fat body and secreted into the haemolymph before they are transported into the oocytes (Hagedorn and Kunkel, 1979). Elevated *Vg* expression is typically found in queens but can also be found in younger inside workers compared to older outside workers (Corona et al., 2007; Wu et al., 2021), potentially linked to the increased oocyte development in inside workers. Moreover, the insect Toll pathway and its receptors play an important role in both, immunity and development (Evans et al., 2006).

In outside workers, we found the overexpression of *dual oxidase isoform X2*, *zinc finger proteins* and *fatty acid synthase*. Dual oxidase induces oxidative stress to challenge gut microbiome bacteria (Ha et al., 2005; Sistermans et al., 2023), while zinc is involved in regulatory pathways (Kandel, 2009; Klug, 2005). Fatty acid synthase, involved in energy metabolism and promoting survival in older animals (Chaudhari and Kipreos, 2018), may help compensate cellular damage from ageing in outside workers, supporting the hypothesis that they were older, consistent with age-related division of labour in social insects.

While we acknowledge that precise control of worker age was not possible in our study, our analysis revealed significant overlaps in upregulated genes between inside workers and young queens, and between outside workers and old queens. This suggests a link between worker location and age. The upregulation of hexamerin storage proteins in inside workers and young queens not only indicates that inside workers were indeed younger than outside workers but also suggests that both groups store proteins to produce reproductive or trophic eggs. Additionally, the greater oxidative stress resistance, the investment in antioxidants, and increased egg-laying potential in inside workers suggest a link between worker age, egg production, and task. In *Temnothorax* ants, worker task is more strongly linked to gene expression than age and ovarian

development (Kohlmeier et al., 2019). Although we used age polyethism (Wilson, 1971) to infer worker age based on location it is also possible that reproductive status influences their location, as workers with higher reproductive potential (i.e., an individual's ability to develop their ovaries) tend to remain inside the nest (Bourke, 1988).

The physiological and molecular influence of ageing in queens

We investigated the effects of ageing on fecundity and tissue-specific gene expression in young and old *T. magnum* queens. We found no evidence that age affected fecundity or brain gene expression, but fat body gene expression varied with age (126 DEGs).

We found a higher expression of *chymotrypsin-2-like, vitellogenin-1-like,* and *vitellogenin-2-like* (C-Vgs; Kohlmeier et al., 2018) in old queens. Chymotrypsin is often expressed as immune response against (bacterial) infection (Viljakainen et al., 2018), possibly linked to an increase in body repair within old queens. In *P. barbatus, vitellogenin 1* expression is higher in queens and nurses, while *vitellogenin 2* is higher expressed in foragers (Corona et al., 2013). These copies might stand in contrast to each other, one indicating a high reproductive potential and the other indicating reproductive senescence in old queens of *T. magnum*. Moreover, the elevated *vitellogenin 2* expressions in both old queens and young workers may reflect a secondary function, such as protection against oxidative stress (Seehuus et al., 2006).

Young queens (and inside workers) overexpressed *hexamerin-like* and *acyl-CoA Delta(11) desaturase-like*. In *Camponotus festinatus* founding queens, high levels of *hexamerin* (storage proteins; Beintema et al., 1994) are related to the production of the first batch of eggs (Martinez and Wheeler, 1994). Since nest spots of *T. magnum* may contain over 350 queens (Seifert et al., 2017), the workers might not provide each queen with the same amount of food and care (Hannonen et al., 2002; Keller, 1988; Chen and Vinson, 2000). Thus, young queens possibly use up their storage proteins to start reproduction. Acyl-CoA desaturases play a crucial role in synthesizing cuticular hydrocarbons (CHCs) by introducing double bonds into fatty acyl-CoA precursors, leading to the formation of unsaturated hydrocarbons (Hazel and Williams, 1990;

Miyazaki and Ntambi, 2003). In ants, these enzymes are integral to producing CHCs, which serve as chemical signals and protective barriers against desiccation. Notably, the genomes of *Linepithema humile* and *Solenopsis invicta* harbour multiple acyl-CoA desaturase genes. This gene expansion may enable these species to diversify their chemical profiles, potentially facilitating adaptation to new ecological niches and contributing to their invasive success (Helmkampf et al., 2015).

Our results contrast with a study on *Temnothorax* ants showing that older queens have better developed ovaries (Negroni et al., 2019). Indeed, differences in queen ovarian development might depend on the age gap between young and old queens (Seistrup et al., 2023). In our study, old queens were at least one year older than young queens. Queen lifespan varies among species and correlates with social structure (Keller and Genoud, 1997; Kramer et al., 2022). In queens of similarly invasive, polygynous species, queen lifespans may range from one to three years (Goodisman and Ross, 1999; Reuter et al., 2001). Thus, *T. magnum* queens have likely shorter lifespans, potentially explaining the similar ovarian development with age. Similarly, the lack of age-related fecundity changes may be characteristic of invasive species (Majoe et al., 2024). Contrarily to previous studies (Negroni et al., 2019; Von Wyschetzki et al., 2015), we did not find many age-related differences in fat body gene expression (~1500 DEGs vs. 126 DEGs). In the supercolonial ant *Lasius neglectus*, similarly subtle age-dependent shifts in queen gene expression were observed (Majoe et al., 2024; 165 DEGs). The limited variation in age-related gene expression may correlate with the species' polygynous nature and a shorter queen life expectancy, suggesting stable fecundity and gene expression throughout life (Jaimes-Niño et al., 2022). However, the lack of precise information on queen life expectancy and the varying durations queens of different ages spent under laboratory conditions could have influenced our findings. While queen physiology may deteriorate more rapidly towards the end of their lives, our queens were likely middle-aged, which may explain the absence of pronounced age-related changes in gene expression and fecundity (Jaimes-Niño et al., 2022).

Conclusion

Our study offers novel insights into the physiological and molecular responses of the supercolonial ant *Tapinoma magnum* across varying social structures, revealing distinct life-history traits compared to non-invasive species (Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021b). We found that workers survived better in monogynous colonies than in polygynous ones, while complete queen removal increased mortality, highlighting the complex interplay between social factors and physiology. Physiological and transcriptional changes in workers may be related to their location and align with patterns seen in other non-invasive ant species (Majoe et al., 2021; Negroni et al., 2021b). The increased oocyte development in inside workers and the shared gene expression profiles with young queens suggests that both, egg-laying potential and age, drive the observed physiological changes, such as enhanced oxidative stress resistance and altered gene expression, though disentangling these factors is challenging. Future research should control for worker age to clarify how location, age, and egg-laying potential affect transcription and stress susceptibility. Contrarily, queen age minimally impacted fecundity and gene expression, suggesting that reproductive activity is maintained with age (Jaimes-Niño et al., 2022).

Author contributions

SF, RL, MM and AL designed the experiment. Ants have been collected by SF, MM, SS, and AL. SS, supervised by SF and co-supervised by AL, conducted the survival and the oxidative stress experiment. AL analysed the data collected by SS, dissected and measured ovaries from workers as well as dissected fat bodies and extracted the RNA. MM and AL dissected brain and fat body from the queens and MM helped AL with the RNA extractions. SF, RL, TJC and MM helped AL in the statistical analyses and interpretation. TJC and AL annotated the transcriptome assembly. AL wrote a first draft, and all authors commented on it. AL, SF, RL, TJC finalized the manuscript based on these comments. SF supervised the experimental parts of the project.

Acknowledgements

We thank Ina Knuf for helping us collect the ants and assisting SS during the experimental phase of the study. We thank Juliane Hartke and Timo Wentong Lin for their help in generating the *de novo* transcriptome assembly. Thanks to Barbara Feldmeyer for her input on the annotation of the *de novo* transcriptome assembly. Moreover, we are grateful to Jenny Fuchs for providing the ant illustrations.

Data accessibility statement

The raw data and code for the statistical analyses, including annotation files are available through Dryad (https://doi.org/10.5061/dryad.j0zpc86p3). Additional supplemental information (methods, figures, tables) can be found in the supplement. Raw read sequences are accessible through the NCBI BioProject ID PRJNA1120839.

Chapter 1

Supplementary Material

Worker survival and egg production– but not transcriptional activity– respond to queen number in the highly polygynous, invasive ant *Tapinoma magnum*

Anna Lenhart, Megha Majoe, Sibel Selvi, Thomas J. Colgan, Romain Libbrecht, Susanne Foitzik

Published as: Lenhart, A., Majoe, M., Selvi, S., Colgan, T.J., Libbrecht, R. and Foitzik, S. (2025), Worker Survival and Egg Production—But Not Transcriptional Activity—Respond to Queen Number in the Highly Polygynous, Invasive Ant *Tapinoma magnum*. Mol Ecol, 34: e17679. https://doi.org/10.1111/mec.17679

Chapter 1

Supplementary Methods

RNA extraction and Sequencing

Large inside and outside workers from each experimental box were sampled after the 58-day survival experiments. The workers were frozen at -80°C between 12:00 and 16:00 and their fat bodies were dissected. We took the fat body from four individual workers and pooled their fat bodies to create 72 individual RNA pools. We created two RNA pools per worker location (inside, outside) across the six cohorts, resulting in a total of 12 replicates per worker location. Each pool of four fat bodies was stored in 100µl of TRIzol™ LS Reagent (Invitrogen™) at -80°C until extraction. RNA was extracted in a 4:1 ratio of TRIzol: Chloroform: Isoamylalcohol followed by purification steps using the Qiagen RNA-easy Mini Kit. At the Beijing Genomics Institute (BGI), the High Sensitivity RNA Analysis Kit (Fragment Analyser) was used to check the quality of the extracted RNA. The libraries were prepared and sequenced by BGI using the Illumina HiseqXTen sequencing platform, yielding 150 bp paired- end reads with a sequencing depth of 23.36 ± 3 (mean ± sd) million reads.

For the queen dataset, we sampled and dissected six young and six old queens (12 total queens in total) and transferred brain and abdominal fat body tissues into 100µl of TRIzol™ LS Reagent (N= 12 brain samples; N=12 fat body samples). RNA extractions were conducted as described above and extracted RNA was checked using the Agilent 2100 Bioanalyzer, RNA 6000 Nano Kit at BGI. Library preparation and sequencing were similarly performed by BGI using the Illumina HiseqXTen sequencing platform, yielding in 150 bp paired- end reads with a sequencing depth of 23.58 ± 2.54 (mean ± sd) million reads.

Head width measurements

To validate our visual size classification of large and small workers, we collected additional 389 workers, categorized as large or small, from inside and outside the source colony. We measured head widths (the distance between the eyes) using Leica LAS software to determine if large workers were significantly larger than small workers. Consistent with the method used in the experiment set-up, workers were classified based on a single visual observation. A Welch's t-test was then performed to assess whether the head widths of large and small workers differed significantly.

Chapter 1

Supplementary Figures

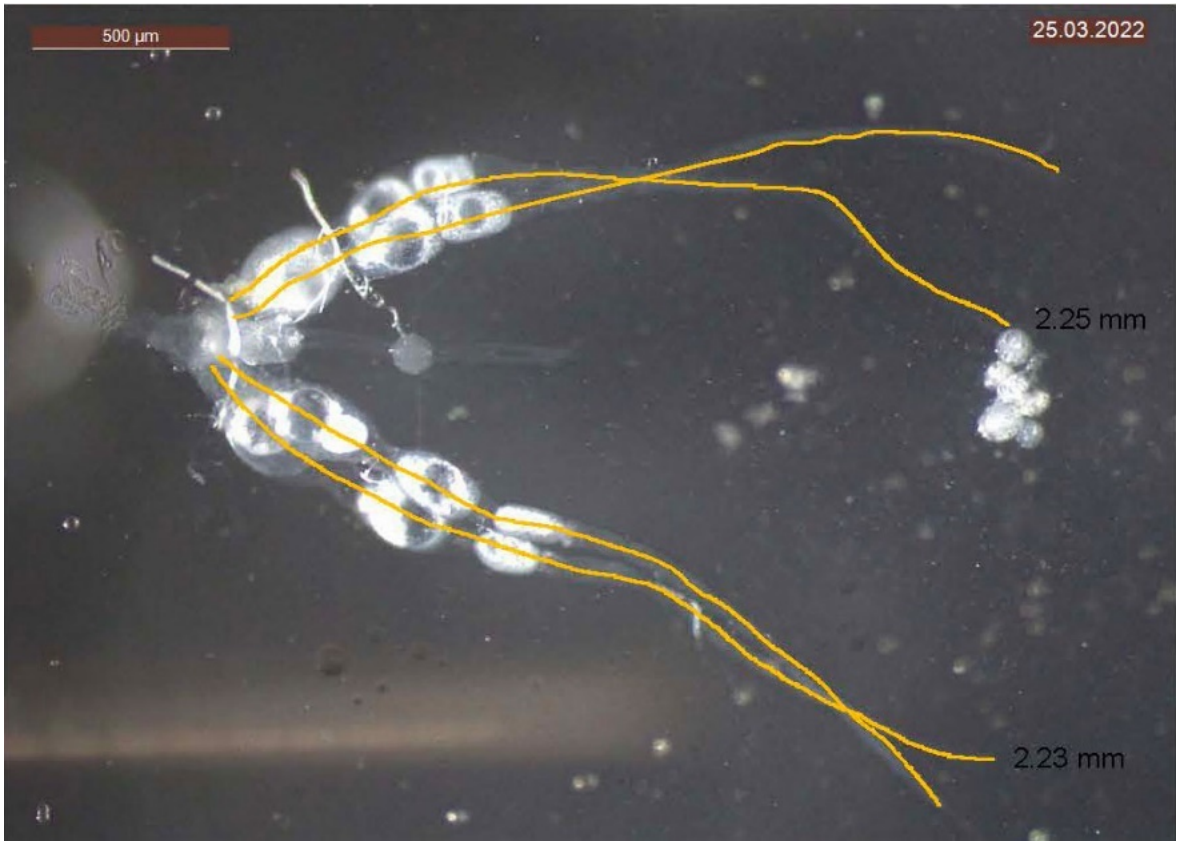

Figure S 1: Image of a worker ovary showing four ovarioles.

The orange lines illustrate the measurement paths for the ovarioles. The lengths of the two longest ovarioles were used to calculate the mean ovariole length. The scale bar is shown in the top left corner, and the dissection date is displayed in the top right corner.

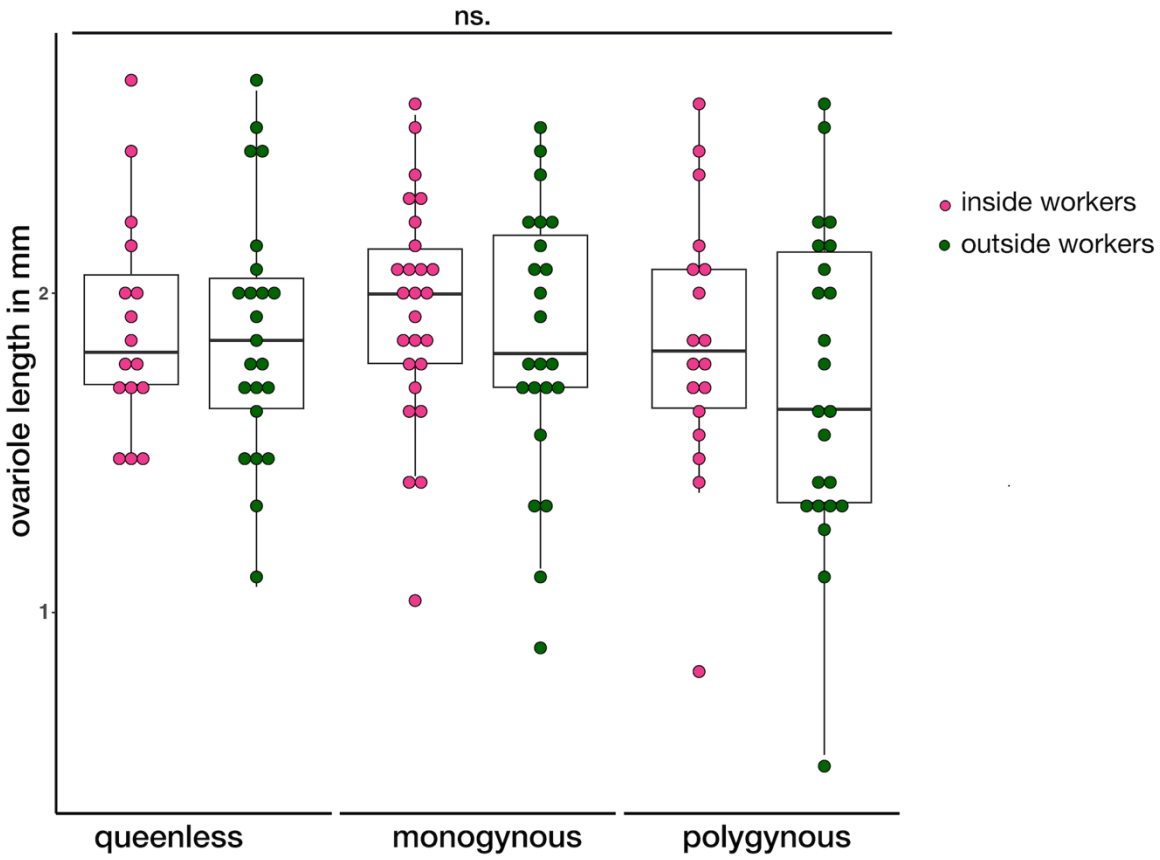

Figure S 2: Mean ovariole length within inside and outside workers of the three queen treatments (queenless, monogynous, polygynous).

Inside and outside workers had similarly long ovarioles, independent to queen number ($LMER_{queen\ number}$: $\chi^2 = 1.17$, $p = 0.57$; $LMER_{worker\ location}$: $\chi^2 = 2.75$, $p = 0.10$).

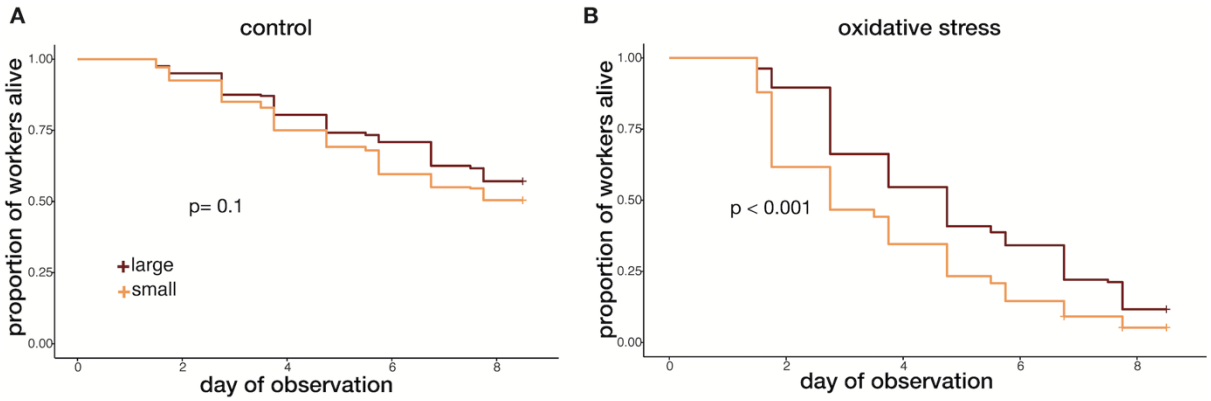

Figure S 3: Worker survival dependent to worker size within the oxidative stress experiment.

A) Large (dark red line) and small (orange line) workers within the control treatment survived similarly long ($\chi^2 = 2.69$, $p = 0.1$). **B)** Large workers did survive better than small workers when subjected to paraquat-induced oxidative stress ($\chi^2 = 46.11$, $p < 0.001$).

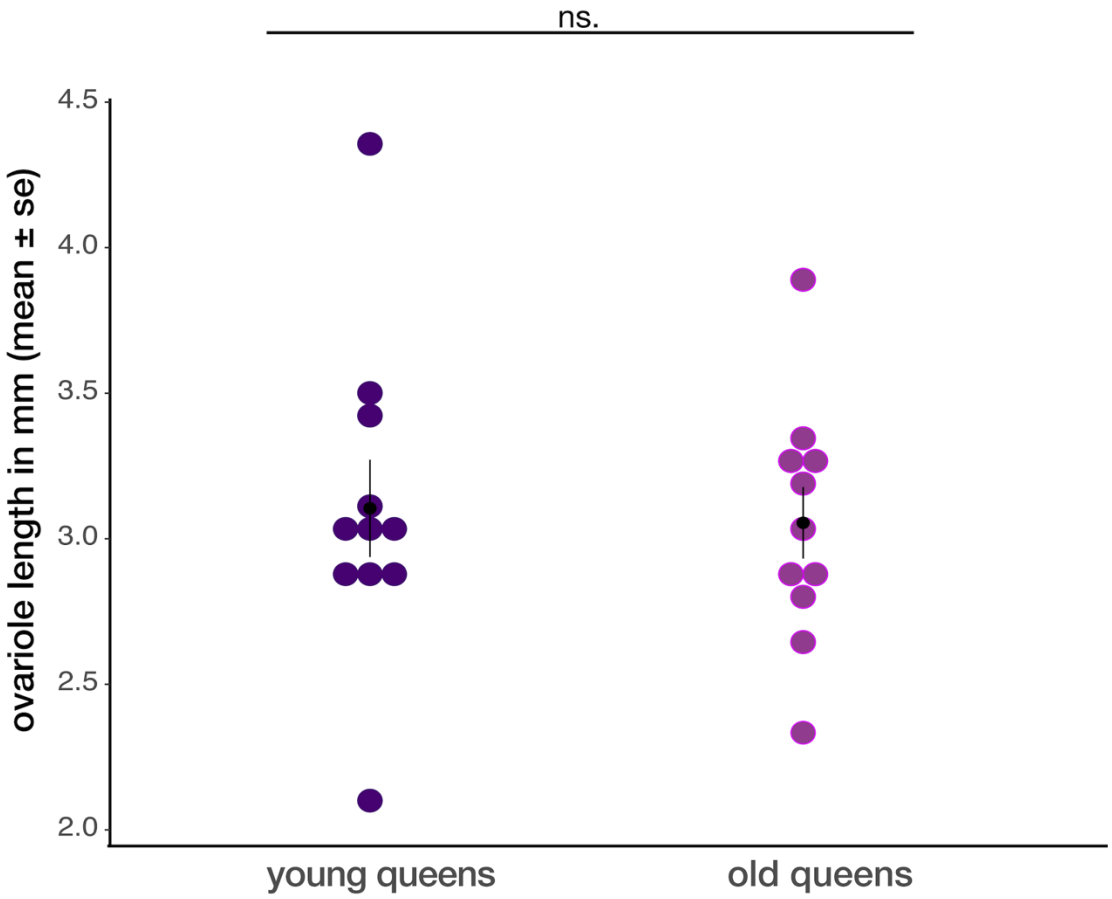

Figure S 4: Mean ovariole length of young and old *Tapinoma magnum* queens.

Young and old queens had similar long ovarioles (LMER: $\chi^2 = 0.02$, $p = 0.811$).

Chapter 1

Supplementary Tables

Table S 1: Significantly enriched GO Terms (molecular function) in the list of genes that were overexpressed in inside workers compared to outside workers including the respective p-values obtained from the Fisher's exact test.

GO-ID	Term	Annotated	Significant	Expected	p-value
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	368	29	6.79	2.1E-09
GO:0005506	iron ion binding	377	25	6.96	3.2E-08
GO:0004497	monooxygenase activity	323	21	5.96	4.8E-07
GO:0005515	protein binding	4385	128	80.93	5.1E-07
GO:0003723	RNA binding	1254	49	23.14	9.9E-06
GO:0005509	calcium ion binding	496	22	9.15	0.00016
GO:0020037	heme binding	437	20	8.07	0.00021
GO:0008017	microtubule binding	131	9	2.42	0.00075
GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	179	8	3.3	0.03658
GO:0005085	guanyl-nucleotide exchange factor activity	139	6	2.57	0.04448

Table S 2: Significantly enriched GO Terms (molecular function) in the list of genes that were overexpressed in outside workers compared to inside workers including the respective p-values obtained from the Fisher's exact test.

GO-ID	Term	Annotated	Significant	Expected	p-value
GO:0005515	protein binding	4385	209	123.95	1.3E-19
GO:0020037	heme binding	437	37	12.35	3.7E-09
GO:0004497	monooxygenase activity	323	31	9.13	4.3E-09
GO:0005506	iron ion binding	377	33	10.66	4.5E-09
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	368	32	10.4	9.2E-09
GO:0005509	calcium ion binding	496	30	14.02	9E-05
GO:0004181	metallocarboxypeptidase activity	36	6	1.02	0.00047
GO:0004842	ubiquitin-protein transferase activity	170	13	4.81	0.00122
GO:0004252	serine-type endopeptidase activity	314	19	8.88	0.00166
GO:0008270	zinc ion binding	975	44	27.56	0.00167
GO:0005085	guanyl-nucleotide exchange factor activi...	139	10	3.93	0.00624
GO:0004674	protein serine/threonine kinase activity	158	11	4.47	0.01036
GO:0004222	metalloendopeptidase activity	207	12	5.85	0.01514
GO:0031267	small GTPase binding	72	6	2.04	0.01614
GO:0140359	ABC-type transporter activity	145	10	4.1	0.01809
GO:0043169	cation binding	2690	136	76.04	0.01945
GO:0016747	acyltransferase activity, transferring g...	446	15	12.61	0.02366
GO:0016298	lipase activity	111	9	3.14	0.03566
GO:0050660	flavin adenine dinucleotide binding	311	13	8.79	0.04072

Chapter 2

Experimental suppression of reproduction reduces lifespan in clonal raider ants

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Submitted to: PNAS (as of February 2026)

Abstract

Longevity is typically traded off with fecundity in most organisms, but social insects seemingly defy this pattern, as the most fecund individuals in the colonies also live the longest. However, confirming this reversal of the fecundity-longevity trade-off requires disentangling reproductive activity from confounding factors such as age, morphology, individual experience, and genetic background. Using clonal raider ants (*Ooceraea biroi*), we experimentally manipulated same-age, monomorphic, and genetically identical workers after adult emergence to either suppress or maintain their reproduction over five months. We tracked survival and reproductive activity throughout the experiment and analysed age-related transcriptomic changes in the brain and fat body. Our key finding is that the experimental suppression of reproduction reduced worker survival compared to both workers with experimentally enhanced reproductive activity and workers naturally cycling between reproductive and non-reproductive phases. Transcriptomic analyses further identified candidate molecular mechanisms linking reproduction and lifespan. As they aged, older ants that were forced to reproduce maintained or increased the expression in the fat body of genes with antioxidant protection, immunity, and detoxification functions. Similarly, brain gene expression patterns indicated that they maintained brain function compared to older workers that were prevented from producing eggs. These findings provide direct experimental evidence supporting the reversed fecundity-longevity trade-off in social insects.

Keywords: Social evolution; Life history evolution; Ageing; Fecundity; Longevity; Social insects.

Chapter 2

Introduction

Understanding the relationship between fecundity and longevity is a central topic in the evolutionary study of life history traits. Of particular interest is how social insects seem to defy the typical trade-off between reproduction and lifespan that is typically found in most other organisms (Heinze et al., 2013; Heinze and Schrempf, 2012; Kramer et al., 2015). The most striking examples of this phenomenon are found in ants and termites, where the individuals that produce eggs (*i.e.*, the queens) can live for decades, while the non-reproductive individuals (*i.e.*, the workers) live only several months to a few years (Carey, 2001; Heinze and Schrempf, 2008; Keller and Genoud, 1997; Korb, 2016). Although the molecular mechanisms underlying caste-specific ageing in social insects remain unclear, previous research has highlighted the crucial role of physiological strategies that control the balance between ageing and fecundity. For example, highly fecund social insect queens may live longer because of modifications in the conserved insulin/insulin-like growth factor signalling (IIS), juvenile hormone (JH), target of rapamycin (TOR) and vitellogenin (Vg) pathways (Corona et al., 2007; Korb et al., 2021; Lin et al., 2021; Monroy Kuhn et al., 2021, 2019; Rodrigues and Flatt, 2016; Yan et al., 2022; Yi et al., 2021) and/or because they shift from prioritizing immunity to producing antioxidants as they age (Negroni et al., 2019).

Beyond caste differences, this reversed trade-off is also found between reproductive and non-reproductive workers. In most species of social Hymenoptera, the workers retain some reproductive potential with the ability to lay haploid, male-destined eggs under specific conditions, for example in the absence of the queen (Bourke, 1988; Wilson, 1971). Previous studies reported that when workers invest in reproduction, they experience an extended lifespan compared to non-reproductive workers (Blacher et al., 2017; Kohlmeier et al., 2017; Kuszewska et al., 2017; Peeters et al., 2000; Tsuji et al., 1996). Moreover, totipotent workers have the ability to mate or reproduce clonally, and thus may fully replace the queen (Peeters, 1991; Rabeling and Kronauer, 2012). In those cases, reproductive activity is likewise associated with an extended lifespan. For instance, reproductive *Harpegnathos saltator* workers (*i.e.*, gamergates) exhibit a five-fold prolonged lifespan compared to their non-reproductive nestmates (Ghaninia et al., 2017; Glastad et al., 2023; Yan et al., 2022). Similarly, in the clonal ant *Platythyrea*

punctata, dominant workers that monopolize reproduction live twice as long as their subordinate sisters (Hartmann and Heinze, 2003; Hartmann et al., 2020; Matte et al., 2024). It has been proposed that the longer lifespan of reproductively active workers can be explained by enhanced resistance to oxidative stress and by changes in transcriptional activity in the brain and fat body compared to non-reproductive nestmates (Glastad et al., 2023; Korb et al., 2021; Majoe et al., 2021; Negroni et al., 2021b; Seehuus et al., 2006; Sheng et al., 2020; Yan et al., 2022).

Social insects are generally sensitive to changes in their social environment. Most studies that reported that fecund workers live longer involved manipulations of their social environments, such as queen removal experiments (Blacher et al., 2017; Kennedy et al., 2021; Kohlmeier et al., 2017; Majoe et al., 2021; Yan et al., 2022). However, even alternative manipulations of colony composition, such as social isolation experiments or pathogen exposures, may similarly impact individual lifespans (Boulay and Lenoir, 2001; Kohlmeier et al., 2016; Lenoir et al., 2001). In *H. saltator*, experimental manipulations of the social environment showed that the lifespan of reproductive workers was increased when helpers were present (Haight and Liebig, 2025). The age and behavioural specialization and age of workers are additional important factors determining their longevity and reproductive potential. In most species, younger workers that remain inside the nest are usually more likely to activate their ovaries, show increased stress resistance and exhibit extended lifespan upon queen loss, compared to older workers that forage outside the nest and face higher chances of extrinsic mortality (Bourke, 1988; Heinze and Schrempf, 2008; Kohlmeier et al., 2019, 2017; Korb et al., 2021; Majoe et al., 2021; Negroni et al., 2021b). Thus, worker age, behavioural specialization and social environment should be controlled for when investigating the association between reproduction and lifespan in social insects.

We argue that previous reports of a positive association between fecundity and longevity in social insects may have been affected by alternative, confounded factors (Blacher et al., 2017). First, comparisons of queens and workers can only provide correlational evidence and cannot prove a causal link between reproduction and lifespan. The queen and worker castes not only differ in reproduction, but also in many other traits such as age, morphology, allometry, genetic background, behaviour, social

environment, immunity, metabolism and diet (Conway, 1986; Hartfelder and Engels, 1998; Kronauer and Libbrecht, 2018; Wilson and Hölldobler, 2005). Any of those factors could underlie caste-specific differences in longevity. Second, the extended lifespan of workers in response to experimental manipulations of worker reproductive activity could also be explained by associated variation in such confounding factors. For example, the age, individual experience and genetic background of workers are typically not controlled for in queen removal experiments. Those factors may drive differences in longevity (Korb et al., 2021; Wyatt et al., 2023; Yamamoto et al., 2022), the likelihood of becoming reproductively active and/or the ability to overcome the potential costs of reproduction (Blacher et al., 2017). Similarly, the difference in longevity between reproductive, dominant individuals and non-reproductive, subordinate individuals may stem from variation in age, genetic background, diet, stress level, metabolism or the care provided by social partners (Bernadou et al., 2020, 2018; Haight and Liebig, 2025; Hartmann and Heinze, 2003).

Some of the past investigations of the relationship between fecundity and longevity in social insects did take measures to partially control for some of the typical confounding factors. For example, studies in ants often use age polyethism and cuticular pigmentation to estimate worker age (Kohlmeier et al., 2018, 2017; Lenhart et al., 2025; Majoe et al., 2021; Seid and Traniello, 2006). However, these methods only provide a broad, relative estimate of age differences, and do not allow a quantitative measurement of individual age. Finally, although potential effects of the colony of origin are typically controlled for in the experimental design and statistical analyses (Kohlmeier et al., 2017; Lenhart et al., 2025; Majoe et al., 2021), most study species cannot be propagated under laboratory conditions, thus limiting the experimental control of an individual's genotype. The clonal raider ant *Ooceraea biroi* is a great study system to investigate the causal link between fecundity and longevity while controlling for age, morphology, individual experience, and genetic background. Colonies of *O. biroi* lack the queen caste and are composed of morphologically similar, genetically identical workers (Kronauer et al., 2013). All workers reproduce clonally and synchronously via thelytokous parthenogenesis, leading to the emergence of discrete age cohorts (Ravary and Jaisson, 2004). These characteristics allow the production of experimental colonies composed of

same-age workers with identical genetic backgrounds and uniform individual experiences (Kronauer et al., 2013; Libbrecht et al., 2018, 2016; Oxley et al., 2014; Teseo et al., 2013; Ulrich et al., 2016). Moreover, *O. biroi* colonies continuously alternate between reproductive and brood care phases (Ravary and Jaisson, 2004).This phasic life cycle is tightly regulated by the presence of larvae that inhibit worker reproduction (Ravary et al., 2006; Teseo et al., 2013). This inhibitory effect of larvae allows the experimental control of the reproductive activity of *O. biroi* workers (Ravary et al., 2006; Ulrich et al., 2016).

Here, we used *O. biroi* as a study system to experimentally investigate the impact of reproduction on longevity. We experimentally manipulated reproductive activity by regularly removing or adding larvae to small experimental colonies composed of same-age, genetically identical workers. We predicted that, if there is a reversed trade-off between fecundity and longevity in *O. biroi*, an experimental suppression of worker reproduction would reduce lifespan and/or an experimental activation of reproduction would extend lifespan. To investigate the molecular basis of the link between fecundity and longevity, we compared the brain and fat body transcriptomes of younger and older individuals between experimental conditions. Our study aimed to experimentally confirm the reversed fecundity-longevity trade-off in social insects and shed light on its underlying molecular mechanisms.

Material and Methods

Set-up of experimental colonies

Ooceraea biroi source colonies used in this study came from the two clonal lines A and B (Kronauer et al., 2012; Libbrecht et al., 2016). We maintained the colonies at 25°C and 80% humidity and fed them with frozen or fresh *Lasius niger* or *Temnothorax nylanderi* brood, depending on availability. We used five source colonies (two from clonal line A and three from clonal line B) to create six cohorts (one source colony was used twice), with each cohort consisting of five experimental colonies. The source colonies were selected based on colony size, the number of newly emerged workers and the presence of brood at the time of the experimental set-up. Each experimental colony consisted of

13 same-age workers with or without larvae, depending on treatment (Fig. 1A). To ensure that all workers were of the same age, we collected newly emerged workers characterized by their light coloration (hereafter referred to as “callows”) when the source colonies were at the onset of the brood care phase (Fig. 1A). Each experimental colony was housed in a small, air-tight plastic box (15 x 10 x 5cm) with a ca. 1.5cm-thick plaster layer at the bottom. Each box contained a single nest site, which consisted in a depression in the plaster covered by a glass slide.

Experimental manipulations of reproductive activity

O. biroi colonies consistently cycle between reproductive and brood care phases (Ravary et al., 2006). During the reproductive phase (ca. 18 days), all workers stay inside the nest and lay eggs, while during the brood care phase (ca. 16 days) workers care for the larvae inside the nest and forage for food (Ravary et al., 2006; Ulrich et al., 2018, 2016). The transitions between phases are regulated by the presence of larvae, which inhibit egg production (Ravary et al., 2006). We used this effect of larvae to experimentally manipulate worker reproductive activity in the experimental colonies (Fig. 1). Three treatments were set up: control (one colony per cohort), forced reproduction (two colonies per cohort), and forced brood care (two colonies per cohort) (Fig. 1A). All experimental colonies (treatment and control) were checked and fed every three days (Fig. 1B). The forced reproduction colonies were set up by grouping 13 same-age callow workers without larvae to stimulate reproduction. We then maintained active worker reproduction by removing all eggs produced every three days, ensuring that no larvae developed that would inhibit reproduction (Fig. 1B). To set up the forced brood care colonies, we grouped 13 same-age callow workers with 13 larvae. We then ensured that worker reproduction remained inhibited by replacing all last-instar larvae (defined as fully white larvae without meconium) with younger larvae every three days, collected from the source colonies (Fig. 1B). We also removed all eggs from the forced brood care colonies. The control colonies were set up by grouping 13 same-age callow workers with 13 larvae. Although the control colonies were then allowed to follow their natural phasic cycle and alternated between reproductive and brood care phases, we removed late-stage pupae (shortly before emergence) every three days to ensure that no new adult

workers emerged (Fig. 1B). We did not remove or replace eggs or larvae in the control colonies. We fed all experimental colonies every three days, at the same time as we conducted the experimental manipulations described above.

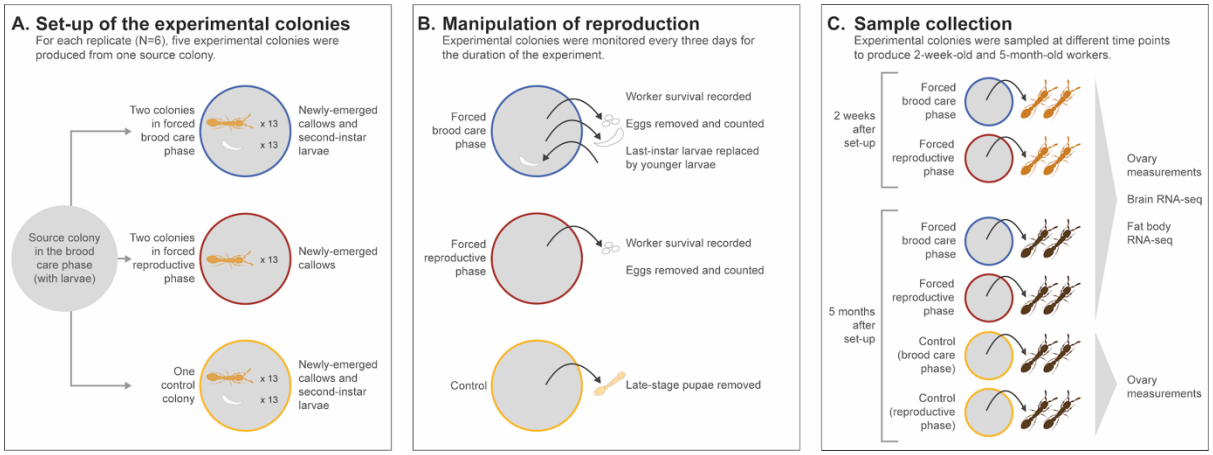

Figure 1: Experimental set-up, monitoring and sample collection.

A) Source colonies (N = 6) in the brood care phase were divided into five experimental colonies, each consisting of 13 same-age callow workers. Two experimental colonies were provided with 13 larvae to produce the forced brood care colonies (blue circle), while two other experimental colonies were kept without brood, producing the forced reproduction colonies (red circle). The fifth experimental colony was provided with 13 larvae and served as the control colony (yellow circle). **B)** Every three days, we monitored all experimental colonies for worker survival and conducted experimental manipulations to produce variation in reproductive activity. To suppress reproduction, we removed all larvae reaching their final instar from the forced brood care colonies and replaced them with younger larvae. To maintain active reproduction in the forced reproduction colonies, we removed and counted all eggs. We did so as well in the forced brood care colonies, when applicable. In control colonies, we removed late-stage pupae to prevent the emergence of new workers. **C)** We sampled workers after 14 days (two 2-week-old workers per experimental colony from four forced brood care and four forced reproduction colonies) and five months (two 5-month-old workers per experimental colony from each of the forced brood care colonies, forced reproduction colonies, control colonies in the brood care phase and control colonies in the reproductive phase). For each of those 48 workers, we conducted ovary measurements. From the workers collected in the forced colonies, we produced 32 individual brain RNA-seq samples and 32 individual fat body RNA-seq samples.

Worker survival and egg production

To investigate the effect of our experimental manipulations on worker survival, we monitored the number of workers every three days throughout the entire experimental period. If more than 50% of the workers in an experimental colony died before the planned end of the experiment, the entire cohort of five experimental colonies was terminated. This happened in two out of our six cohorts. These two cohorts were included in our survival analyses, and the surviving workers were integrated as censored data points (Clark et al., 2003). However, these two cohorts were excluded from the gene expression and reproductive activity analyses (Fig. 1). To further verify that our experimental manipulations affected egg production in the forced phases, we counted the number of eggs that were removed every three days from the experimental colonies (Fig. 1B).

Sample collection

To investigate the effect of worker age and the experimental manipulations on transcriptional and reproductive activity, we collected samples at different time points in the experimental colonies (Fig. 1C). We aimed to collect younger and older workers in the forced reproduction and forced brood care colonies, and older workers in the control colonies. For each cohort, we used one forced reproduction and one forced brood care experimental colony to produce younger workers, which were sampled 14 days after the set-up of the experimental colonies (Fig. 1C). Since the younger age cohort was sampled before their first cycle could be completed, we did not include a control colony for the 2-week-old workers. For each cohort, we used the remaining three experimental colonies (one control, one forced reproduction, and one forced brood care colony) for our older worker samples, which were sampled five months after the set-up of the experimental colonies (*i.e.*, 5-month-old workers; Fig. 1C). Our preliminary experiments indicated that mortality increased after five months of experimentally manipulating worker reproductive activity. Thus, we decided to set the experimental period to a maximum of five months to ensure that the number of workers would be sufficient for sample collection. Samples were flash frozen in liquid nitrogen between 12:00 and 16:00 and stored at -80°C. Older workers from the control colonies were sampled at two time points

(Fig. 1C). Depending on the phase the workers were in after five months, we sampled two workers either in the brood care or reproductive phase. We then monitored the control colonies until they reached the respective other phase to sample two additional workers. For the analyses of reproductive activity and gene expression, we only used workers from the four cohorts that went through the entire planned period of the survival experiment (14 days for the 2-week-old workers and five months for the 5-month-old workers) (Fig. 1C).

Reproductive activity measurements

Worker ovaries were dissected in 1x PBS under a stereomicroscope (Leica S9i, Microsystems CMS GmbH, Wetzlar, Germany), and pictures of the ovaries were taken using Leica LAS software (version 4.12.0). For each ant, we measured three variables to gauge their reproductive activity: the mean of the length of the two ovarioles, the mean of the surface area of the two largest oocytes in development, and the number of oocytes in development (Ulrich et al., 2016). Ovariole length and oocyte area were measured with the ImageJ Fiji software (version 2.9.0).

RNA sequencing

For each of the four cohorts that reached the end of the experimental period, we dissected the brain and fat body from two randomly chosen workers collected in the forced reproduction (2-week-old and 5-month-old; Fig. 1C) and forced brood care (2-week-old and 5-month-old; Fig. 1C) experimental colonies. For each individual, we first dissected the brain in 1x PBS, followed by the fat body. Dissections were conducted on a glass slide positioned on a petri dish filled with a mix of ice and dry ice. This resulted in 32 brain and 32 fat body samples (Fig. 1C). Each tissue was stored in 100 µl of TRIzol reagent (Invitrogen) at -80°C until extraction. Fat bodies were homogenized shortly before RNA extraction using sterile pestles. To extract RNA, we added to each sample400 µl of TRIzol and 100 µl of UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v; Invitrogen). Tubes were flicked gently and incubated on ice for 5 minutes. After centrifugation (15 min, 12,000 ×g, 4 °C), the upper aqueous phase was transferred

to a new 1.5 ml Eppendorf tube, and the transferred volume from each sample was recorded. Ice-cold 100% Isopropanol (50% of the noted volume) was added, mixed and the solution was loaded onto a RNeasy Mini spin column (Qiagen RNeasy Mini Kit). Columns were incubated for 1 min on ice, then centrifuged (1 min, 11,000 ×g, 4 °C). RNA was washed with 400 µl and then 300 µl of Buffer RPE (Qiagen RNeasy Mini Kit), each followed by centrifugation (1 min, 11,000 ×g, 4 °C). A final dry spin was performed in a new collection tube (1 min, 11,000 ×g, 4 °C). RNA was eluted in 30 µl RNase-free water (Qiagen RNeasy Mini Kit), incubated at room temperature for 1 min, and centrifuged (1 min, 11,000 ×g, 4 °C). Eluted RNA was kept on ice, sealed, and stored at –80 °C. The quality check (Agilent 2100 Bioanalyzer), library preparation (NEBNext Ultra RNA library preparation kit) and sequencing (Illumina Novaseq 6000, PE 150bp, 9Gb per sample) of the 64 RNA samples were performed by Novogene (Cambridge, UK), yielding a sequencing depth of 31.47 ± 4.08 (mean ± sd) million reads.

Statistical analyses of survival and reproductive activity

To investigate the effect of the experimental manipulations on worker survival, we used the *coxme* package version 2.2-22 (Therneau, 2024) in RStudio (R version 4.4.1, R Core Team, 2024) to build cox-regression mixed-effect models for the survival experiment. We did not include in this analysis the forced reproduction and forced brood care experimental colonies that were sampled two weeks after set-up. We included treatment (forced reproduction, forced brood care or control) as a fixed factor, and cohort and source colony of origin as random factors.

To investigate the effect of the forced reproduction and brood care treatments on the number of eggs removed from the experimental colonies, we used a linear mixed effect model (*lme4* package, version 1.1-33; Bates et al., 2015) to explain the number of eggs removed in a one-month period divided by the number of workers present in the experimental colonies at the end of that month) by the treatment (forced reproduction or forced brood care), the month (one to five) and their interaction. Experimental colony was included as random factor. To analyse worker reproductive activity, we built for each of the three measurements of reproductive activity (mean ovariole length, mean oocyte

area, oocyte number) a linear mixed-effect model (*lme4* package) that included treatment (control or forced) as fixed factor and the colony identifier and cohort of each experimental box as random factor. We ran separate analyses to investigate differences in reproductive activity between reproduction and brood care phases in 2-week-old workers, 5-month-old workers kept in forced phases, and 5-month-old workers from the control experimental colonies.

For all models, we used the 'Anova' function from the package *car* version 3.1-2 (Fox et al., 2024) to test the effect of explanatory variables and the package *emmeans* version 1.10.2 (Lenth, 2024) to perform post-hoc pairwise comparisons.

Differential gene expression and functional enrichment analyses

The differential gene expression analysis included the 32 fat body RNA-seq samples and 32 brain RNA-seq samples. We trimmed sequence adaptors using Fastp version 0.2 (Chen et al., 2023) and performed quality check using FastQC version 0.11.8 (Andrews, 2010). We mapped the RNA sequencing (RNA-seq) reads to the *Ooceraea biroi* genome (Ensembl Metazoa Obir_v5.4) and quantified transcript abundances using Kallisto version 0.50.1 with default parameters (Bray et al., 2016). Differential gene expression analysis was conducted in RStudio (R version 4.4.1). Transcript abundances imported into an estimated count matrix with the package *tximport* version 1.32.0 (Soneson et al., 2016) were used as input for differential gene expression analysis using the *DESeq2* package version 1.44.0 (Love et al., 2014). Subsequent analyses were conducted separately for the brain and fat body samples. We removed genes with zero reads in more than four replicates from the count matrices, resulting in 11,333 genes for the brain samples and 11,358 genes for the fat body samples.

We ran separate differential expression analyses to investigate a priori defined questions. The first objective of the analysis was to identify genes whose expression was affected by an interaction between worker age and the forced phase (forced brood care and forced reproduction). We used the LRT function of *DESeq2* to compare the following models:

Full model: ~cohort + forced_phase + age + forced_phase:age

Reduced model: ~cohort + forced_phase + age

The second objective was to test the main effect of the forced phase. We did so by comparing the following models:

Full model: ~cohort + forced_phase + age

Reduced model: ~cohort + age

The third objective was to test the main effect of age. We did so by comparing the following models:

Full model: ~cohort + forced_phase + age

Reduced model: ~cohort + forced_phase

To interpret the lists of genes whose expression was affected by an interaction between two variables, we conducted a cluster-based analysis with the *DEGreport* package version 1.40.1 (Pantano, 2017) using default parameters (only for lists with at least 30 genes) to extract genes with similar expression patterns. To further identify gene annotation and function and Gene Ontology (GO) term annotation, we used Ensembl Metazoa Biomart to create annotation files for each list of differentially expressed genes. We identified enriched GO terms for molecular functions and biological processes by performing individual GO term enrichment analyses for gene lists with at least 400 genes. To do so, we implemented Fisher's exact tests with the package *TopGo* version 2.56.0 (Alexa and Rahnenführer, 2024) using the weight01 algorithm. The scripts used for the GO term enrichment analyses were originally developed by Colgan et al. (2019).

Results

The experimental suppression of reproductive activity decreased worker survival

To investigate whether reproduction influences survival in clonal raider ants, we experimentally manipulated workers to either constantly reproduce (forced reproduction colonies) or to remain in a non-reproductive brood care phase (forced brood care colonies) over five months. We also included a treatment where experimental colonies followed the natural phasic cycle (control colonies). We found that worker survival differed among treatments ($\chi^2 = 19.35$, $p < 0.0001$, Figure 2A). Workers forced to reproduce continuously survived better than workers forced to remain in the brood care phase ($z = 3.8$, $p = 0.0004$, Figure 2A), but similarly to workers from the control colonies ($z = 1.28$, $p = 0.407$, Figure 2A). Workers forced to stay in the brood care phase survived less well than workers that were allowed to follow their typical phasic cycle in control colonies ($z = 2.99$, $p = 0.008$, Figure 2A).

We noted that from the six cohorts of experimental colonies, two cohorts exhibited a sudden drop in survival in the forced brood care colonies around 80 days after the experimental set-up. To investigate whether the effect of the experimental manipulations on worker survival was solely driven by these two cohorts, we reran the survival analysis after excluding them from the data set. Despite losing one-third of the biological replicates, we still found weak evidence that workers forced to reproduce survived better than workers forced to remain in the brood care phase ($z = 2.2$, $p = 0.07$, Supplementary Figure S1).

The experimental manipulations affected egg production

To investigate whether the effect of the forced phases on worker survival was driven by physiological changes in reproductive activity, we counted the number of eggs removed from the experimental colonies every three days for the five months of the experiment. We counted 145.25 ± 21.7 (mean ± sd) eggs in the forced reproduction colonies and only 16.5 ± 3.04 eggs in the forced brood care colonies, confirming that the experimental

manipulation of worker reproductive activity was effective. To further analyse whether the number of eggs laid depended on the month of the experiment (month 1 to 5) and the number of workers still alive in the experimental colonies, we calculated the mean number of eggs per worker for each month. We found strong evidence that the mean number of eggs was higher in forced reproduction colonies compared to forced brood care colonies ($\chi^2 = 89.17$, $p < 0.00001$; Figure 2B). Additionally, the number of eggs per worker varied among the months of the experiment ($\chi^2 = 21.02$, $p = 0.0003$; Figure 2B), likely due to a sharp decrease in egg number in the last month of the experiment (Figure 2B; Supplementary Table S1). This monthly variation was likely driven by an interaction between the forced phase and the month ($\chi^2 = 12.46$, $p = 0.014$). We found more eggs per worker in the forced reproduction colonies compared to the forced brood care colonies for the first ($t = 5.31$, $p = 0.0004$), second ($t = 5.68$, $p = 0.0001$), third ($t = 3.75$, $p = 0.022$), and fourth ($t = 5.09$, $p = 0.0007$) month, but not for the last (fifth) month of the experiment ($t = 1.37$, $p = 0.93$) (Figure 2B).

The absence of evidence for a difference in egg production between forced reproductive and brood care phases in the fifth month was confirmed by our measurements of ovariole length, oocyte size, and the number of oocytes in development. We found no evidence that older workers experimentally manipulated to stay in the reproductive or brood care phase for over five months differed in ovariole length ($\chi^2 = 0.2$, $p = 0.65$), oocyte size ($\chi^2 = 0.1$, $p = 0.75$; Figure 2C), or oocyte number ($\chi^2 = 0.64$, $p = 0.42$). Similarly, younger workers forced to stay in the reproductive or brood care phase for 14 days did not differ in ovariole length ($\chi^2 = 0.02$, $p = 0.89$), oocyte size ($\chi^2 = 0.18$, $p = 0.67$) or oocyte number ($\chi^2 = 0.62$, $p = 0.43$). We also performed measurements of ovarian and egg development in the control colonies to compare reproductive and brood care phases. Although we expected strong differences (Ravary et al., 2006; Teseo et al., 2013; Ulrich et al., 2016), we found only moderate evidence that 5-month-old workers collected in the reproductive phase had longer ovarioles than those of the same age collected in the brood care phase ($\chi^2 = 4.46$, $p = 0.035$). The oocyte size ($\chi^2 = 1.91$, $p = 0.17$) and the number of oocytes in development ($\chi^2 = 1.3$, $p = 0.25$) did not differ between phases (Supplementary Figure S2).

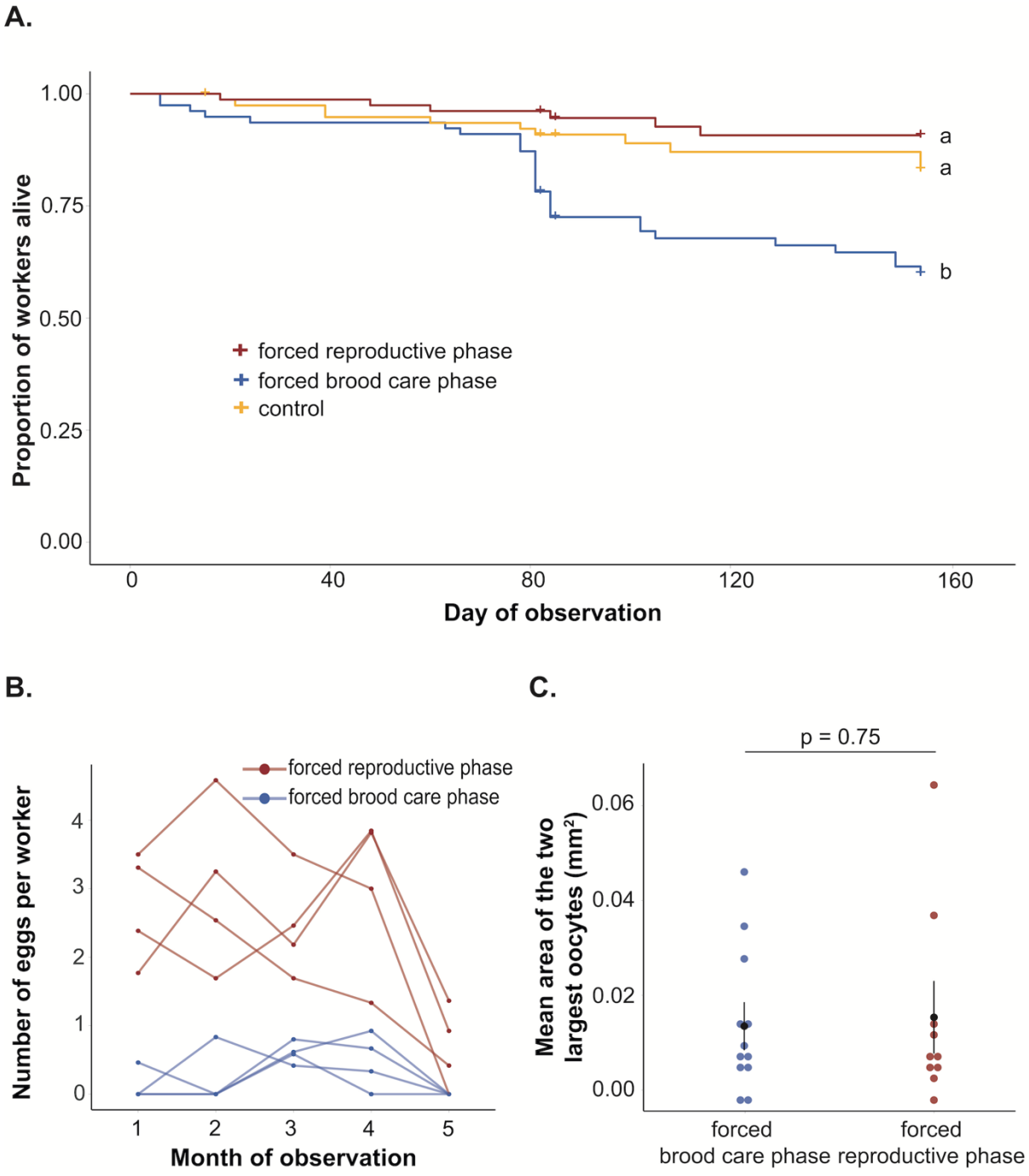

Figure 2: The influence of the experimental manipulation of reproductive activity on survival and physiology in 5-month-old *O. biroi* workers.

A) Workers from the forced reproduction colonies (red line) showed no difference in survival compared to non-manipulated workers from the control colonies (yellow line; $z = 1.28$, $p = 0.407$). Both, workers in the forced reproductive phase and in the control, survived better than workers inhibited from reproduction in forced brood care colonies (blue line; forced brood care phase vs. control: $z = 2.99$, $p = 0.008$, forced brood care phase vs. forced reproductive phase: $z = 3.8$, $p = 0.0004$). **B)** The mean number of eggs was higher in forced reproduction colonies (red lines) compared to forced brood care colonies (blue lines; $\chi^2 = 89.17$, $p < 0.00001$). The number of eggs per worker differed between the months (1 to 5) of the experiment

(χ^2 = 21.02, p = 0.0003) with a sharp decrease in egg number in the fifth month, while there was an interaction between the forced phases and the month (χ^2 = 12.46, p = 0.014). Workers in the forced reproductive phase laid more eggs per worker than in the forced brood care colonies for the first (t = 5.31, p = 0.0004), second (t = 5.68, p = 0.0001), third (t = 3.75, p = 0.022), and fourth (t = 5.09, p = 0.0007) months, but not for the fifth month (t = 1.37, p = 0.93). **C)** The mean area (mean±se) of the two largest oocytes did not differ between 5-month-old workers in the forced reproductive and forced brood care phases (χ^2 = 0.1, p = 0.75).

Different age-associated gene expression changes between workers in experimentally forced phases

To investigate the molecular basis of the differential survival, we compared the age-associated gene expression changes between individuals in forced reproduction and forced brood care colonies. We hypothesized that genes underlying the differences in survival would show differences in expression between 2-week-old and 5-month-old individuals in one forced phase, but not – or differently – in the other. Thus, we first focused on genes showing a significant interaction between the forced phases and age.

In the brain, we found 13 genes whose expression was affected by such an interaction (Figure 3A-B; Supplementary Figure S3; Supplementary Table S2). The gene *protein hu-li tsi shao* (adjusted p = 0.0043) was more expressed in 5-month-old workers from forced reproduction colonies, while its expression did not depend on age in workers from forced brood care colonies (Figure 3A). We found similar expression profiles for genes with annotations related to zinc ion binding, oxidoreductase activity (*e.g.*, *synaptic vesicle membrane protein VAT-1 homolog-like*; adjusted p = 0.038; Figure 3B) and lipid metabolic processes (*e.g.*, *apolipophorin*; adjusted p = 0.038; Supplementary Figure S3; Supplementary Table S2).

In the fat body, we found that the interaction between forced phases and age influenced the expression of 54 genes (Supplementary Table S3), 49 of which could be grouped into two clusters based on this interaction. The first cluster consisted of 19 genes that showed decreased expression with age in forced brood care but increased expression with age in forced reproduction (Figure 3C). Within the list of differentially expressed genes in the first cluster, three genes were annotated with processes related

to oxidoreductase activity, such as *peroxiredoxin-6* (adjusted p = 0.002, Figure 3E). Inspection of the second cluster, which contained 30 genes, indicated a decreased expression with age in the forced brood care colonies but a more stable expression with age in the forced reproductive colonies. Of these, we found three genes with annotations involved in oxidoreductase activity (*e.g.*, *aldehyde dehydrogenase, dimeric NADP-preferring, transcript variant X8*, adjusted p = 0.008), and two genes with annotations involved in proteolysis (*e.g.*, *angiotensin-converting enzyme*, adjusted p = 0.02, Figure 3F). Moreover, we found one gene with annotations associated with DNA repair mechanisms (*uncharacterized LOC105280125*, adjusted p = 0.01), and another with detoxification functions (*UDP-glucuronosyltransferase 2C1, transcript variant X3*, adjusted p = 0.007; Ahn and Marygold, 2021).

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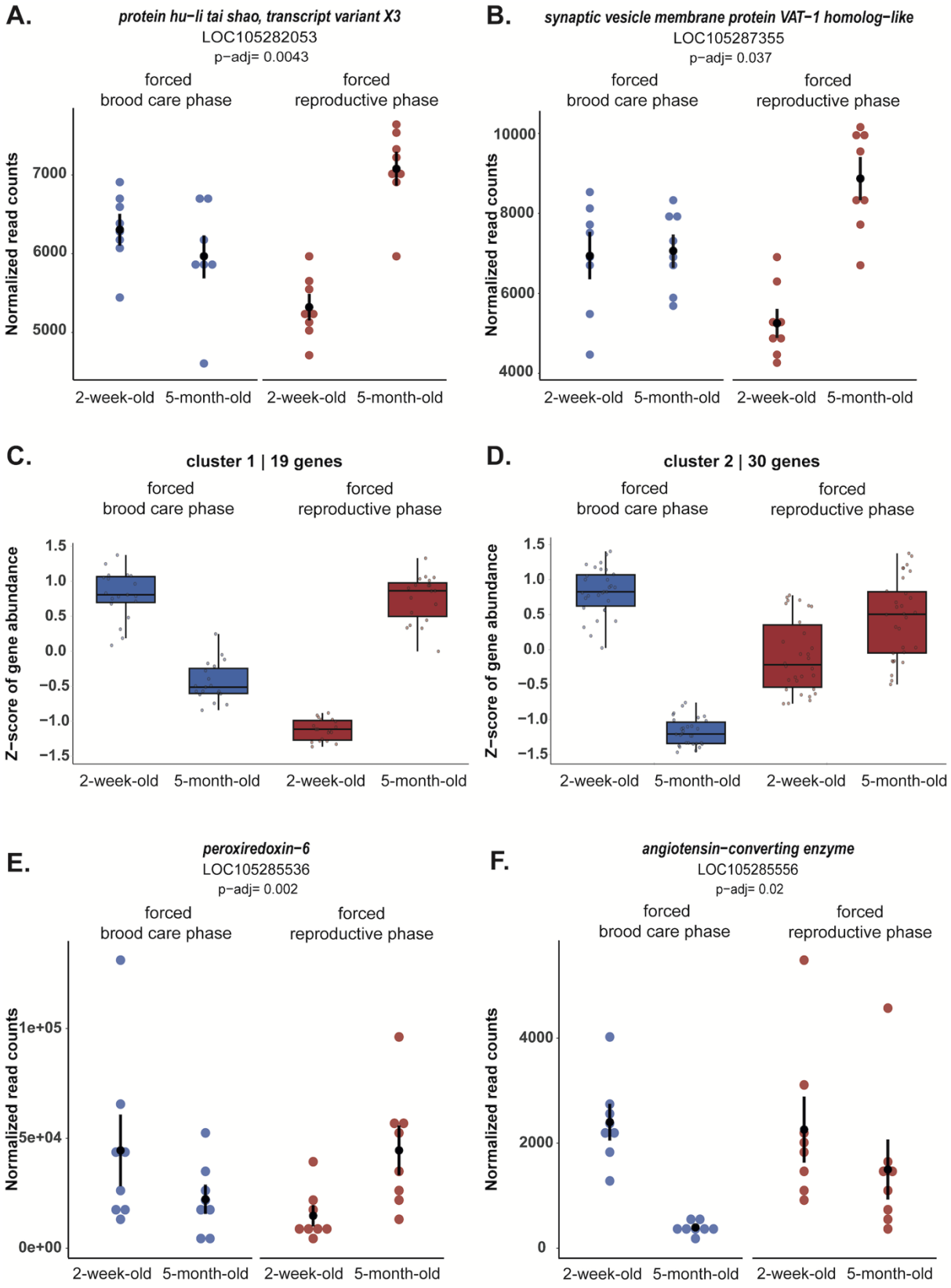

Figure 3: Age-associated transcriptomic changes depended on the experimental manipulation of reproductive activity.

A-B) The brain expression (mean±se) of two genes of interest was affected by an interaction between age and forced phases. **C-D)** 49 of the 54 genes whose expression in the fat body was influenced by an

interaction between age and forced phases grouped into two clusters. **E-F)** The fat body expression (mean±se) of two genes of interest was affected by an interaction between age and forced phases.

Age-independent transcriptomic differences between workers in experimentally forced phases

In addition to genes that showed distinct age-associated patterns between forced phases, we hypothesized that the differential survival may also be regulated by genes that were differentially expressed between forced phases, independent of age. In the brain, we identified 67 genes that were differentially expressed between the forced reproductive phase and the forced brood care phase (Supplementary Table S4). Of these, 27 genes (40.3%) were upregulated in the forced reproductive phase, and 40 genes (59.4%) were upregulated in the forced brood care phase. In the fat body, we identified 59 differentially expressed genes, including 25 genes (42.4%) upregulated in the forced reproductive phase and 34 genes (57.6%) in the forced brood care phase (Supplementary Table S4).

Genes upregulated in the forced reproductive phase were annotated with functions associated with DNA binding (*e.g., class A basic helix-loop-helix protein 15*, adjusted p = 0.005), DNA repair (*e.g., uncharacterized LOC113563224, transcript variant X4*, adjusted p = 0.011), dioxygenase activity (*e.g., phytanoyl-CoA dioxygenase domain-containing protein 1 homolog*, adjusted p = 0.01) and antioxidant activity, including the removal of superoxide radicals (*e.g., protein asteroid*, adjusted p = 0.018) (Supplementary Table S4). In the fat body, genes that were upregulated in workers from the forced reproductive phase were annotated with functions associated with proteolysis (*e.g., venom serine protease*, adjusted p = 0.038), dioxygenase and oxidoreductase activity (*e.g., prolyl 3-hydroxylase 2, transcript variant X1*, adjusted p = 0.002), and DNA binding (*e.g., LIM domain only protein 3*, adjusted p = 0.003) (Supplementary Table S4).

Genes that were overexpressed in the brain of workers from the forced brood care phase were annotated with functions associated with protein phosphorylation (*e.g., MAP/microtubule affinity-regulating kinase 3, transcript variant X2*, adjusted p < 0.0001),

RNA binding (*e.g., piwi-like protein Siwi*, adjusted p = 0.014) and monooxygenase activity (*e.g., peptidylglycine alpha-hydroxylating monooxygenase*, adjusted p = 0.041) (Supplementary Table S4). Among the genes that were upregulated in the fat body of workers from the forced brood care phase, we identified genes that were associated with functions related to response to stress (*e.g., heat shock 70 kDa protein cognate 4, transcript variant X1*, adjusted p = 0.038), proteolysis (*e.g.*, *serine protease snake*, adjusted p = 0.023) and oxidoreductase activity (*e.g., cytochrome P450 4c3*, adjusted p = 0.021) (Supplementary Table S4).

Large transcriptomic changes with age, irrespective of the experimental manipulation

We investigated whether brain and fat body gene expression changed with age by comparing 2-week-old workers and 5-month-old workers. In the brain, we found 2,466 genes with age-related expression differences: 1,237 genes (50.2%) were upregulated in 2-week-old workers, while 1,229 genes (49.8%) were upregulated in 5-month-old workers (Supplementary Table S5). Genes upregulated in 2-week-old workers were enriched for 10 GO terms for molecular functions and 17 GO terms for biological processes (Supplementary Table S6). We found the strongest confidence for enrichment for the GO term oxidoreductase activity (GO:0016491, p = 0.0002), which was associated with 68 of the genes upregulated in 2-week-old workers (*e.g.*, *protoporphyrinogen oxidase*, adjusted p < 0.00001). Genes upregulated in 5-month-old workers were enriched for 20 GO terms for molecular functions and 17 for biological processes (Supplementary Table S6). Here, we found the strongest evidence for enrichment for the molecular function protein serine/threonine kinase activity (GO:0004674, p < 0.00001), while protein binding (GO:000551, p = 0.0002) was associated with highest number of genes upregulated in older workers (262 genes). Further, six genes were associated with the biological process nervous system development (GO:0007399, p = 0.0127).

In the fat body, we detected 430 genes whose expression was influenced by age: 237 genes (55.1%) were upregulated in 2-week-old workers and 193 genes (44.9%) were upregulated in 5-month-old workers (Supplementary Table S5). Genes upregulated in 2-

week-old workers were enriched for five GO terms for molecular functions and one GO term for biological processes (GO:0005975, carbohydrate metabolic process, p = 0.03) (Supplementary Table S6). We found the highest level of confidence for enrichment for the GO term calcium ion binding (GO:0005509, p < 0.00001). One representative for this GO term was *SPARC (secreted protein acidic and cysteine rich*, adjusted p = 0.0002). Genes upregulated in 5-month-old workers were significantly enriched for six GO terms for molecular functions and one for biological processes (GO:0055085, transmembrane transport, p = 0.007) (Supplementary Table S6). In addition to iron ion binding (GO:0005506, p = 0.0024) and monooxygenase activity (GO:0004497, p = 0.0094), 10 genes were associated with oxidoreductase activity (GO:0016705, p = 0.0127; GO:0016614, p = 0.0365). Additionally, we found seven genes with annotations related to protein phosphorylation (e.g., *mitogen-activated protein kinase 7*, adjusted p < 0.00001) (Supplementary Table S5).

Discussion

The typical trade-off between fecundity and longevity that can be observed in most living organisms (Gems and Riddle, 1996; Partridge et al., 1987; Westendorp and Kirkwood, 1998) appears to be absent in social insects: fertile queens live longer than non-reproducing workers (Heinze et al., 2013; Heinze and Schrempf, 2012; Kramer et al., 2015) and workers that become reproductive extend their lifespans (Kohlmeier et al., 2017; Korb et al., 2021; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021b). However, comparing queens and workers or experimentally manipulating worker reproduction via queen removal makes it challenging to disentangle reproductive activity from confounding factors such as age, morphology, individual experience and genetic background (Kohlmeier et al., 2018, 2017; Lenhart et al., 2025; Majoe et al., 2021; Seid and Traniello, 2006). In this study, we used the clonal raider ant *Ooceraea biroi* to investigate the impact of reproduction on lifespan, as well as its transcriptomic basis, while controlling for all these confounding factors.

Our main finding is that the experimental suppression of worker reproduction shortened lifespan: *O. biroi* workers forced to remain in the non-reproductive brood care

phase exhibited a decreased survival compared to workers that were forced to continuously reproduce or to workers from the control colonies that naturally alternated between reproduction and brood care phases. This result cannot be explained by associated variation in age, morphology, individual experience or genetic background, as our experimental design allowed us to control for these typical confounding factors. Thus, we provide experimental validation of the reversed fecundity-longevity trade-off in social insects.

One specificity of this study is that our experimental manipulations of reproduction included both a continuous suppression and a continuous stimulation of reproductive activity, which were compared to workers naturally alternating between reproductive and non-reproductive phases. This experimental design revealed that inhibiting reproduction shortened lifespan, whereas enhancing it did not extend worker survival beyond that of control colonies, suggesting that alternating between reproductive and non-reproductive phases is sufficient to confer the longevity benefits of reproduction. These findings highlight the importance of expanding investigations of the association between fecundity and longevity beyond the usual comparisons of reproductive and non-reproductive individuals (Heinze et al., 2013; Heinze and Schrempf, 2012; Kennedy et al., 2021; Kohlmeier et al., 2017; Korb, 2016; Korb et al., 2021; Kramer et al., 2015; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021b), and call for more nuanced, better controlled manipulations of reproductive activity.

Our transcriptomic analyses identified age-associated variation in gene expression that differed between workers in the forced reproduction and forced brood care treatments. We found 13 genes in the brain and 54 genes in the fat body that exhibited this expression pattern and thus may underlie the decreased survival in response to our experimental inhibition of reproduction. Most of the genes in the fat body (Figure 3C-D) displayed a decreased expression with age in the forced brood care phase, but either no change or an increase in expression with age in the forced reproductive phase. In the brain, 9 of the 13 genes showed such an increase in expression with age in the forced reproductive phase, combined with a decreasing or stable expression pattern in the forced brood care phase (Figure 3A-B; Supplementary Figure S3). These expression patterns indicate that older individuals from the forced reproductive phase may have

maintained and/or upregulated specific pathways, which enabled their longer lifespan. In particular, age-related gene expression changes in both brain and fat body implied an upregulation of molecular pathways involved in body maintenance, cellular repair, and oxidative stress mitigation.

In the brain, genes that showed increased expression with age in workers from the forced reproduction phase were annotated with functions related to neuromotor activity, signalling, lipid transport and metabolic processes (Supplementary Table S2, Supplementary Figure S3). This may indicate that workers that were experimentally forced to reproduce for five months were better at maintaining brain functions and/or expressed higher levels of brain activity as they aged compared to workers with experimentally inhibited reproduction. This was best illustrated by the expression patterns of the gene *hu-li tai shao* (*hts*), coding for a protein involved in reproduction (Yue and Spradling, 1992) and neuromotor function (Kruer et al., 2013), as well as a gene coding for apolipophorins. Apolipophorins are carrier proteins that bind lipids and facilitate their transport from tissue to tissue in animals (Zhao et al., 2020). Beyond their role in immune responses when expressed in the fat body of insects (Zdbicka-Barabas and Cytrynska, 2013), their expression in insect brains - particularly in the glial cells of the lamina - has been suggested to support neuronal activity and maintain ionic and metabolic homeostasis (Bogerd et al., 2000; Coles and Tsacopoulos, 1981; Pentreath et al., 1986; Treherne and Schofield, 1981)

In the fat body, genes that showed different age-related expression changes between the forced brood care and forced reproduction phases were largely associated with functions such as antioxidant protection, immunity, DNA repair, detoxification and oxidoreductase activity (Supplementary Table S3). One example is *peroxiredoxin-6*, which encodes peroxiredoxins. These antioxidant proteins protect insects from ROS-induced damage (Chen et al., 2020). The upregulation of *peroxiredoxin-6* with age in workers from the forced reproductive phase may reflect an enhanced antioxidant protection that could be due to increased metabolic demands and/or physiological stress associated with reproductive activity. Another gene of interest codes for the angiotensin-converting enzyme (ACE), a zinc metallopeptidase that is commonly found in the haemolymph and reproductive tissues of insects (Ekbote et al., 1999, 2003a; Isaac

et al., 1999) and plays a role in immunity (Bernstein et al., 2013) and the regulation of insect fecundity (Ekbote et al., 2003b).

In addition to phase-specific, age-associated expression changes, we propose that genes regulating survival differences between the forced brood care and forced reproduction phases may also show age-independent expression differences between phases. In the brain and/or fat body, workers from the forced reproductive phase showed an upregulation of genes with functions in DNA repair, antioxidant activity and cellular maintenance, suggesting that reproductive activity is associated with enhanced molecular protection (Supplementary Table S4). By contrast, workers from the forced brood care phase overexpressed genes linked to protein phosphorylation, RNA binding, and stress responses (Supplementary Table S4). This indicates that reproductive activity may activate pathways that promote somatic repair and oxidative stress resistance, while the experimental inhibition of reproduction may induce physiological stress that could negatively impact survival.

Although the survival rate was still relatively high at the end of the experiment and the maximum lifespan of *O. biroi* workers has not been quantified experimentally, several lines of evidence indicate that a duration of five months was sufficient to capture changes associated with senescence. First, we observed a drop in egg production in the fifth month for the forced reproduction treatment, which dropped down to the levels continuously observed in the forced brood care ants. Second, our data suggests that egg-laying may also be reduced after five months in non-manipulated workers that follow their typical phasic life cycle, as reproductive activity only differed marginally between phases in the control colonies, contrasting with the well-documented phase-specific reproductive activity that was described repeatedly in younger *O. biroi* individuals in previous studies (Ravary et al., 2006; Teseo et al., 2013; Ulrich et al., 2016). Third, we identified vast gene expression differences between two-week-old and five-month-old workers in both the brain and the fat body that were consistent with transcriptomic changes associated with ageing.

For instance, our enrichment analyses revealed higher oxidoreductase activity in the brains of younger workers, which is consistent with the higher metabolic demands

and energy production needed during early adult life (Kim et al., 2010), as well as a more efficient detoxification of reactive oxygen species (Ekeoma et al., 2023). This aligns with the finding in other ant species that younger individuals are more resistant to oxidative stress than older individuals (Kramer et al., 2021; Majoe et al., 2021; Negroni et al., 2021b). In contrast, genes overexpressed in older *O. biroi* workers were enriched for processes related to the activity of serine/threonine kinases, which are key components of the insulin signalling pathway that regulates central functions such as nutrition, fertility, and longevity in animals (Giannakou and Partridge, 2007; Hung et al., 2012; Korb et al., 2021; Qiu et al., 2024; Tissenbaum and Ruvkun, 1998). Apart from this link to the IIS pathway, we did not identify age-associated expression changes of genes associated with other major conserved pathways such as JH, TOR, or Vg (Corona et al., 2007; Korb et al., 2021; Lin et al., 2021; Monroy Kuhn et al., 2021, 2019; Rodrigues and Flatt, 2016; Yan et al., 2022; Yi et al., 2021). Other enriched terms included protein binding, RNA binding and structural constituents of ribosomes, indicating that the brains of older individuals invested more into the production and stability of proteins, potentially in response to age-related disruption of protein synthesis, folding and degradation (Hipp et al., 2019).

Genes overexpressed in the fat body of younger individuals were enriched for molecular functions associated with cell signalling (*e.g.*, calcium ion binding) (Berridge et al., 2000), while another enriched term was related to chitin-related processes (*e.g.*, chitin binding), reflecting that the cuticle formation of younger, light-coloured individuals was not finalized yet (Hartmann et al., 2019; Merzendorfer and Zimoch, 2003). Our finding of an upregulation of genes associated with oxidoreductase activity, monooxygenase activity, iron ion binding and heme binding in the fat body of older workers indicates an increased need for managing oxidative stress and protection against cellular damage (Ekeoma et al., 2023; Mandilaras et al., 2013; Puig et al., 2017) and may reflect an enhanced need to invest into body maintenance with age (Negroni et al., 2019). These changes may be associated with the decreased egg production in both forced phases in the last month of the experiment.

We acknowledge that there may be alternative explanations for the phenotypic and transcriptomic differences between younger and older *O. biroi* workers. First, our

experimental design involved frequent manipulations of the experimental colonies, to which older individuals were exposed for longer than younger ones. However, it is important to note that survival remained relatively high in all treatments despite these experimental manipulations. Second, although all experimental colonies were handled similarly and at the same rate, we cannot exclude that the level of stress produced by our manipulations could have differed between treatments, as we removed different types of brood items depending on the treatment: only pupae for control colonies; eggs and final instar larvae for colonies in the forced brood care phase; only eggs for colonies in the forced reproductive phase. Third, our manipulation of the presence of larvae not only affected the reproductive activity of workers, but also their behaviour, as they provided care and food to the larvae (Ravary et al., 2006). However, given the small size of the experimental boxes, which limited foraging opportunities, the consistent food provision across all treatments, and the lower survival observed in individuals forced into the brood care phase compared to control colonies that also cared for larvae, we argue that the most parsimonious explanation for the effect of our experimental manipulations on worker survival is that it stemmed from its impact on worker reproductive activity rather than behavioural modifications.

Conclusion

Our study demonstrates that the typical trade-off between fecundity and longevity is reversed in the clonal raider ant *Ooceraea biroi*. By experimentally manipulating the reproductive activity of same-age, monomorphic, and genetically identical workers, we controlled for all typical confounding factors that previous studies on other social insect species could not (Kohlmeier et al., 2018, 2017; Lenhart et al., 2025; Majoe et al., 2021; Seid and Traniello, 2006). Our findings support the reversal of the fecundity-longevity trade-off in social insects, as suggested by previous comparisons of reproductive and non-reproductive individuals (Blacher et al., 2017; Dixon et al., 2014; Hartmann and Heinze, 2003; Heinze et al., 2013; Jaimes-Niño et al., 2022; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2019; Negroni et al., 2021b), and identify molecular mechanisms that likely mediate the link between reproduction and lifespan. Finally, our study indicates that the positive

association between fecundity and longevity in *O. biroi* stems from lifespan being reduced by suppressed reproduction, rather than extended by enhanced reproductive activity.

Author contributions

AL, MM, VN, SF and RL designed the experiment. AL conducted the experiments and analysed the data with help from SF and RL. AL and RL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. SF and RL supervised the study.

Funding

This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) grants NE1969/4-1 (VN), FO 298/26-1 (SF) and LI 3051/3-1 (RL) (from the research unit FOR-2281), and the GRK GenEvo 2526/1 – Project number 407023052 (SF and RL).

Acknowledgements

We thank Martin Coulm and Ina Knuf for their support during the experimental phase of this study.

Data accessibility statement

The code and input data for all analyses are provided as Supplementary Information. Supplementary Tables can be found here. The raw RNA-seq data used in this study is publicly available from the NCBI BioProject [to be completed upon acceptance].

Chapter 2

Supplementary Material

Experimental suppression of reproduction reduces lifespan in clonal raider ants

Anna Lenhart, Megha Majoe, Volker Nehring,
Susanne Foitzik, Romain Libbrecht

Submitted to: PNAS (as of February 2026)

Chapter 2

Supplementary Figures

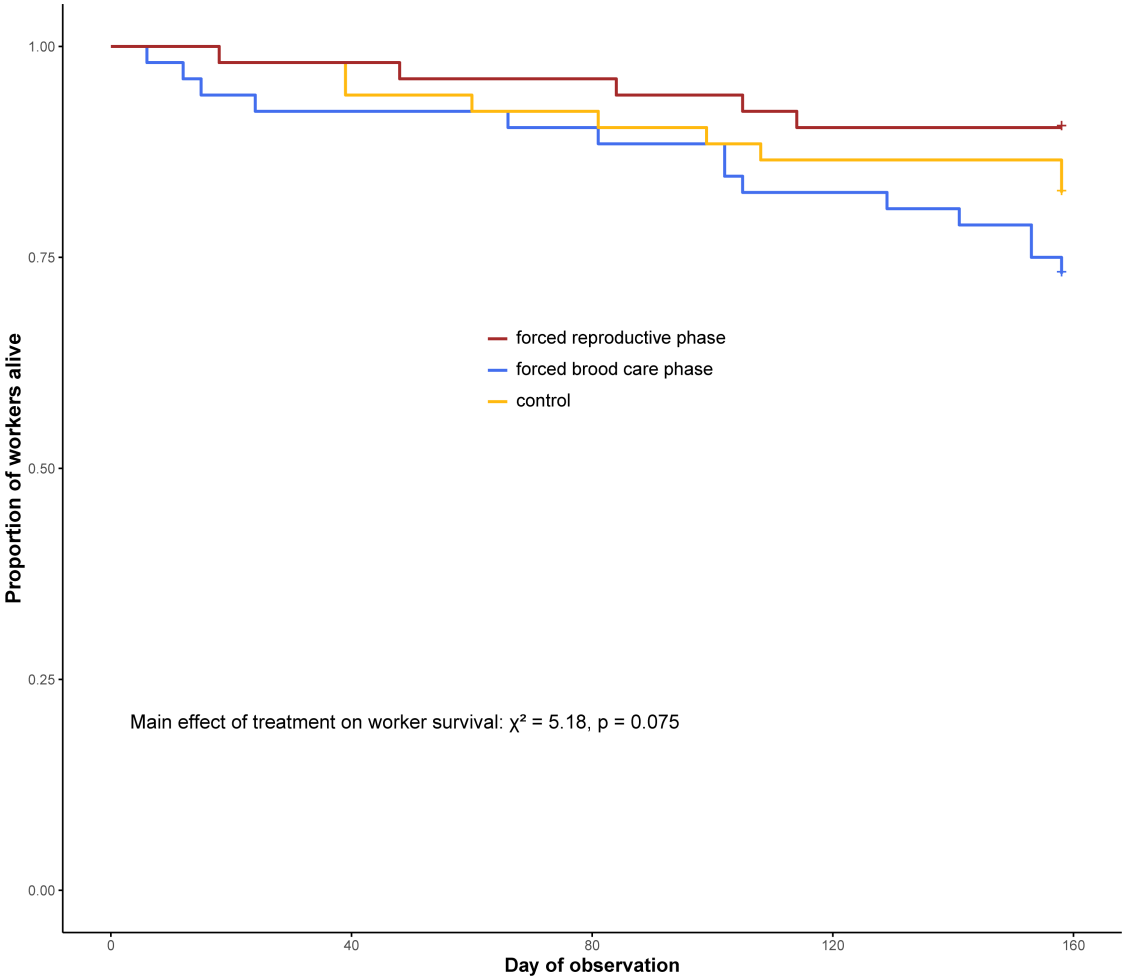

Figure S 1: The influence of the experimental manipulation of reproductive activity on survival after removing the two cohorts with early mortality from the analysis. Workers from the forced reproduction colonies (red line) showed no difference in survival compared to non-manipulated workers from the control colonies (yellow line; z= 1.1, p= 0.51). We found weak evidence that workers forced to reproduce survived better than workers from the forced brood care phase (blue line; z= 2.2, p= 0.07). Worker survival did not differ between workers from the control and forced brood care phase (z= 1.25, p= 0.42).

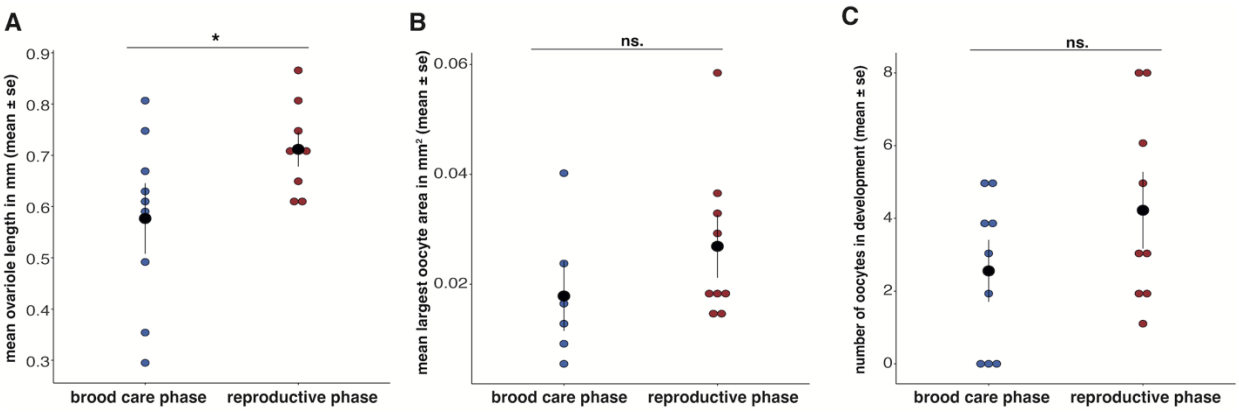

Figure S 2: Limited evidence for differences in ovarian development between 5-month-old control workers in the reproductive and brood care phases. A. Workers in the reproductive phase had longer ovarioles compared to workers in the brood care phase (χ^2 = 4.46, p= 0.035). B-C. We found no evidence that workers in the reproductive and brood care phases differed in B. the mean area of the two largest oocytes (χ^2 = 1.91, p = 0.17) and C. the number of oocytes in development (χ^2 = 1.3, p= 0.25).

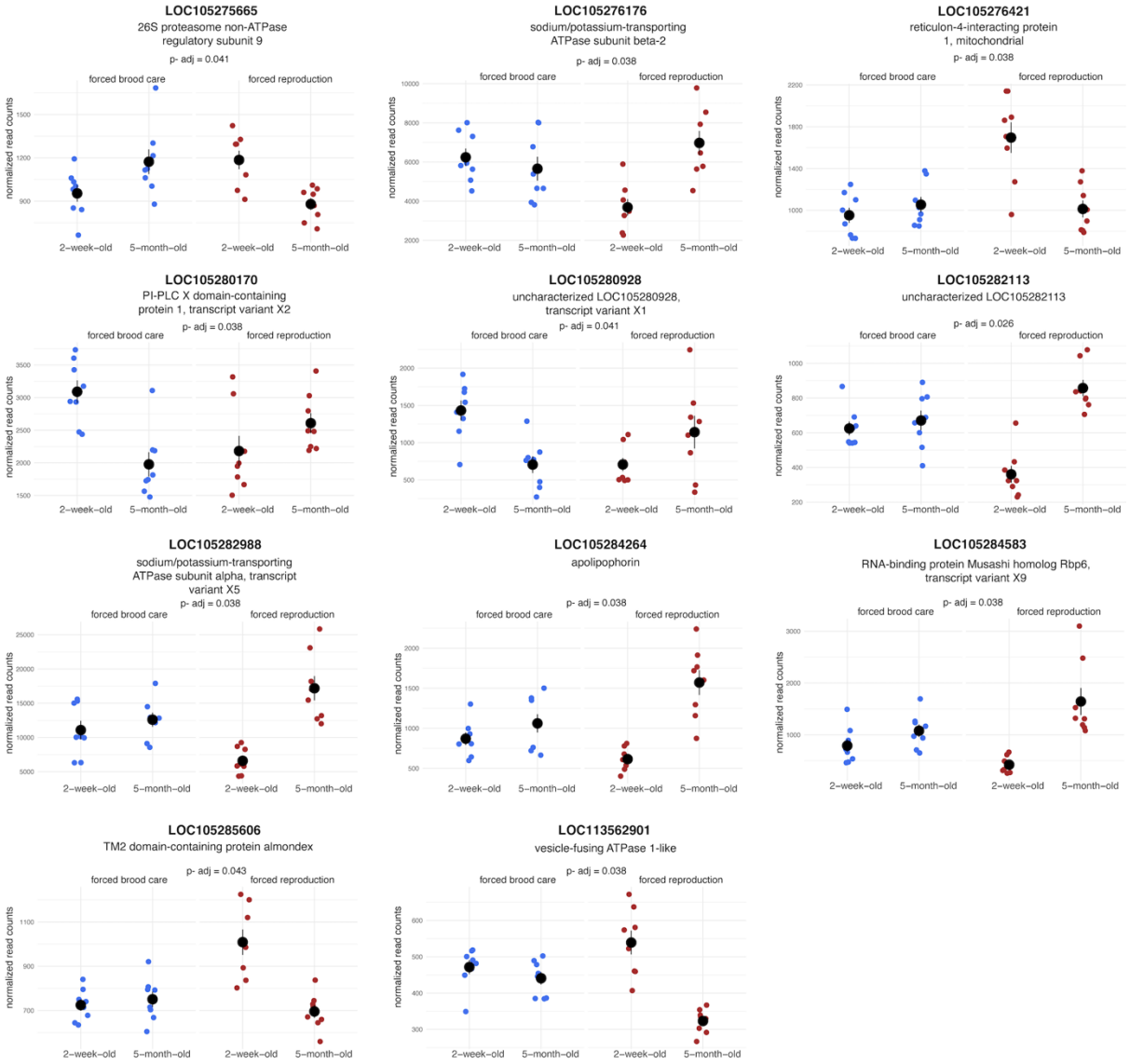

Figure S 3: The brain expression (mean±se) of 13 genes was affected by an interaction between age and forced phases. Two of the 13 genes are presented in Figure 3A-B, and the remaining 11 genes are presented in this figure.

Chapter 3

The impact of queen loss on worker survival and reproduction in the ant *Messor capitatus*

Anna Lenhart, Bianca Todorovic
and Susanne Foitzik

Unpublished manuscript

Abstract

Life-history traits vary markedly within and among social insect societies. In ants, workers are typically short-lived and sterile, whereas queens are highly fertile and long-lived. However, in many species workers can activate their ovaries following queen loss. Such reproductive activation is often associated with increased worker lifespan, but the extent to which this relationship holds across species remains unclear. Here, we investigated the effects of queen loss on worker survival and reproduction in the European seed-harvesting ant *Messor capitatus*, a species in which workers have been reported to reproduce via thelytokous parthenogenesis. We monitored worker survival for 60 days in queenright and queenless sub-colonies. Queen removal did not affect overall worker survival, but survival differed strongly among sub-colony size categories. Post-hoc analyses further revealed sub-colony size-dependent survival responses to queen removal. Queenless workers survived better than queenright workers in large sub-colonies, while the direction of this effect was reversed in medium and small sub-colonies, where queen absence increased worker mortality. Moreover, queen loss consistently increased ovarian development and oocyte production, with reproductive investment further being shaped by worker caste, body size, and sub-colony size. Despite pronounced ovarian activation, only a minority of queenless sub-colonies initiated egg production, and none of the worker-laid eggs developed into adult workers. These findings indicate that in *M. capitatus* queen loss triggers reproductive activation in workers but does not confer consistent survival benefits or lead to successful worker reproduction. This suggests that reproductive investment in this species is decoupled from long-term fitness benefits and is strongly modulated by social and demographic context.

Keywords: Thelytoky, reproduction, social insects, lifespan, life history trade-off

Chapter 3

Introduction

Social insects live in societies with high relatedness, consisting of individuals with varying lifespans that take on different tasks within the colony. In these societies, obligate reproductive division of labour is a key characteristic. Queens (and kings in termites) are the only individuals that can reproduce, while workers perform all other tasks in the colony, such as foraging, nest defence, and brood rearing. In non-social organisms, reproduction is typically traded off with lifespan; organisms that invest more in reproduction usually live shorter lives (Flatt, 2011; Mockett and Sohal, 2006). In social insects, this trade-off is seemingly reversed, as queens are extremely long-lived and reproductively active, whereas workers are usually sterile and live shorter (Carey, 2001; Heinze and Schrempf, 2008; Page and Peng, 2001). Even though most workers are unable to mate, in the absence of the queen, some workers can develop their ovaries and lay haploid eggs that develop into males (Bourke, 1988). However, worker reproduction in queenright colonies is rare, as workers are often policed by their nestmates if they start egg-laying in the presence of the queen (Dijkstra et al., 2010; Ratnieks et al., 2006; Stroeymeyt et al., 2007).

If the queen dies in hymenopteran societies, the residual lifespan of a colony is limited to that of the remaining workers since no more female brood can be produced. Workers may then rear the remaining brood of the queen and subsequently switch to direct reproduction (Dijkstra and Boomsma, 2007; Fletcher and Ross, 1985; Strätz and Heinze, 2004). However, this option is not available to all workers of all ant species. The ability of workers to develop their ovaries is influenced by their age and their task within the colony (Bourke, 1988). Typically, younger workers that stay inside the nest and care for the brood are more likely to develop their ovaries compared to older workers that take on riskier tasks outside the nest and are therefore more prone to extrinsic mortality, a phenomenon known as age polyethism (Bourke, 1988; Giraldo and Traniello, 2014). On the other hand, worker reproductive activity also varies strongly among ant species. Worker sterility is commonly found in species with large colonies, particularly in invasive ant species and those that exhibit strong morphological caste differentiation (Aron et al., 2001; Dijkstra and Boomsma, 2006; Korb and Heinze, 2016; Kronauer et al., 2009). While some ants exhibit worker sterility and many exhibit arrhenotokous parthenogenesis, *i.e.,*

the asexual production of males (Crozier and Pamilo, 1996), a few have evolved worker totipotency via thelytokous parthenogenesis, allowing workers to produce females. These workers can fully replace the queen and in some of these species, the queen caste has been lost secondarily (Peeters, 1991; Rabeling and Kronauer, 2012). Thelytoky has been reported in 51 species among eusocial bees, wasps and ants (Rabeling and Kronauer, 2012). Out of these, infrequent worker reproduction in queenless colonies occurs in 20 ant species out of mainly two subfamilies (Formicinae, Myrmicinae, Rabeling and Kronauer, 2012).

Recent studies on bees, termites and ants showed that the onset of worker reproduction can influence worker lifespan. In fact, workers that start reproduction in the absence of the queen, often live longer than workers that do not reproduce (Dixon et al., 2014; Hartmann and Heinze, 2003; Kohlmeier et al., 2017; Kuszewska et al., 2017; Majoe et al., 2021). Moreover, worker reproduction has a strong impact on worker immunity and gene expression, as they become more resistant to oxidative stress and invest more into antioxidant genes than non-reproductive workers (Kohlmeier et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021b). Similarly, in the clonal ant *Platythyrea punctata* workers that become dominant and take over reproduction live longer than their subordinate sisters (Hartmann and Heinze, 2003). In the wood-dwelling termites *Cryptotermes secundus* totipotent immatures live longer and invest in anti-ageing pathways (Monroy Kuhn et al., 2019; Rau and Korb, 2021). Thus, the classic longevity/fecundity trade-off is not only reversed in social insect queens but also in workers. However, less is known about the impact of queen loss on workers that can reproduce via thelytokous parthenogenesis.

Here, we used the European seed-harvesting ant *Messor capitatus*, a species where workers are reportedly totipotent in the absence of the queen (Grasso et al., 2000), to investigate whether and how queen loss influences worker survival and worker reproduction. To date, there is no evidence that worker reproduction in this species is correlated with increased lifespan. We expected increased worker survival after queen loss, linked with increased worker reproduction and better ovarian development in orphaned colonies (Kohlmeier et al., 2017; Lopes et al., 2020; Majoe et al., 2021). In accordance with the age poylethism that is commonly found in social insects, we

expected younger inside workers to have better developed ovaries than older outside workers (Kohlmeier et al., 2017; Majoe et al., 2021). Moreover, workers of *M. capitatus* are polymorphic and contain major, medium and minor workers (Retana and Cerdá, 1994). Thus, we additionally investigated whether worker morph (major and medium) had an influence on the workers' ovarian development dependent to queen presence as well as worker location (i.e., inside/ outside). We expected major workers to have generally less well developed ovaries as we expected them to be more specialized in food processing and nest defence (Pie and Tschá, 2013), while we predicted smaller medium-sized workers to activate ovarian development more strongly in the absence of the queen. Lastly, we aimed to investigate whether thelytokous worker reproduction can be observed in our experiment, similarly to the reported observation by Grasso et al., (2000).

Material and Methods

Species purchase and laboratory maintenance

Newly mated queens of *Messor capitatus* were ordered from "Fourmishome", traders from Nîmes, France (https://www.fourmishome.fr). They were collected after the nuptial flight in September 2020 and 2021 in Castellbell i el Vilar and delivered to our laboratory at the Johannes Gutenberg University in Mainz in October 2020 (30 queens) and 2021 (25 queens). Founding queens were individually kept in glass tubes (10 cm length x 1 cm diameter) which served as nest site. The tubes were half filled with water that was blocked by cotton. The queens from both collection years went into hibernation at 12 °C from October 2021 until February 2022 to facilitate independent colony founding. Thirteen queens successfully founded their colonies and were thereafter kept at 24°C, with a humidity of 75%. Each colony was housed in plastic boxes (18 cm x 24 cm x 9.5 cm) coated with Fluon® (Whitford GmbH, Diez, Germany) to prevent the ants from escaping the nest. The ground of each box was covered with a ~3 cm thick layer of sand and the glass tubes were placed into each box. The colonies were fed every second week with canary bird seeds, dry cat food and water ad libitum.

Experimental manipulations of queen presence

To investigate whether worker survival depended on queen presence, we equally divided our 13 source colonies (mean number of workers per source colony 55.15 ± 30.14 sd) into equal queenless and queenright counterparts (Supplementary Table S1). Sub-colonies were further categorized by size as small (< 30 workers), medium (< 70 workers), or large (≥ 70 workers; Supplementary Table S1). By equally distributing major, medium, and minor inside and outside workers to each sub-colony, we ensured the most natural conditions for each colony half. We categorized workers as majors, mediums, and minors based on their relative sizes observed in a single observation. The sub-colonies were provided with larvae, while pupae were removed before emergence. Since it has previously been reported that queenless workers of *M. capitatus* can lay female offspring via thelytokous parthenogenesis (Grasso et al., 2000), we predicted that workers in queenless colonies would start reproduction once the queen has been removed, possibly increasing worker survival (Kohlmeier et al., 2017; Lopes et al., 2020; Majoe et al., 2021). Therefore, we aimed to gauge worker egg production in queenless colony halves. To do so, we did not provide the colonies with queen-laid eggs to ensure that eggs laid in queenless colonies would stem from workers. Worker survival was monitored twice per week for 60 days by removing and counting dead workers from each experimental box. We decided on a period of 60 days for our experiment, as we expected that if thelytokous worker reproduction indeed occurred, we would observe the first workers to emerge after 55 days (Grasso et al., 2000). During the monitoring period, the occurrence of worker-laid eggs and their further development was documented.

Measurements of ovarian and egg development

To examine whether ovarian and egg development in workers depended on queen presence as well as worker location (as a proxy for worker age and behavioural caste) and size (major and medium-sized workers), we sampled two inside workers (one major and one medium-sized) and two outside workers (one major and one medium-sized) after the 60-day survival experiment. Workers were flash frozen and stored at -80°C until ovary dissection. Worker ovaries were dissected in Millipore water under a stereomicroscope (Leica S9i, Microsystems CMS GmbH, Wetzlar, Germany) and

pictures of the ovaries were taken using Leica LAS software (version 4.12.0). We measured ovariole length with ImageJ2 Fiji software (version 2.9.0) and calculated the mean of the two ovariole lengths. Similarly, using the Fiji software, we counted the number of developing oocytes in the ovarioles.

Statistical analyses

To investigate the effect of queen presence on worker survival, we used the *coxme* package version 2.2-18.1 (Therneau, 2024) in RStudio (R version 4.2.3, R Core Team, 2024) to build cox-regression mixed-effect models for the survival experiment. We included the treatment (queenless/ queenright) as fixed factor. To account for differences in colony size, we included sub-colony size category (small: < 30 workers, medium: < 70 workers, large: ≥ 70 workers; Supplementary Table S1) as a fixed effect in the survival models. Colony identity was included as a random factor to control for non-independence of workers originating from the same source colony. Hypothesis testing was performed using the 'Anova' function from the package *car* version 3.1-2 (Fox et al., 2024). The package *ggplot2* version 3.4.2 (Wickham, 2016) and *survminer* version 0.5.1 (Kassambara et al., 2025) was used to plot the Kaplan-Meier survival curves. To further quantify specific survival differences, we conducted post-hoc contrasts using the package *emmeans* version 1.10.2 (Lenth, 2024) on a Cox proportional hazards model with identical fixed effects but without random effects (function coxph, package *survival* version 3.8-3; Therneau, 2024). These post-hoc analyses included (i) simple-effect contrasts testing queen presence within each sub-colony size category and (ii) pairwise comparisons among sub-colony size categories averaged over queen presence. We adjusted the p-values for multiple testing using the Benjamini–Hochberg (BH) procedure.

To analyse ovarian and egg development of workers, we modelled the two measured variables (mean ovariole length, oocyte number) using linear mixed-effect models from the package *lme4* (version 1.1-33; Bates et al., 2015) including the treatment (queenless/ queenright), worker location (inside/ outside), worker body size (major/ medium) and sub-colony size category (small/ medium/ large) and all possible interactions therein as fixed factors. Colony identity was included as random factor. We

tested the effect of the fixed factors with the 'Anova' function (*car* package). When significant interactions were detected, we conducted post-hoc pairwise comparisons using the package *emmeans* to compare (i) differences between worker size classes within each sub-colony size category for ovariole length, and (ii) differences between worker locations within each sub-colony size category for oocyte number. For these post-hoc analyses we used the BH-procedure to adjust the p-values for multiple testing.

Results

Queen presence alone had no impact on worker survival

To investigate the effect of queen presence on worker survival, we created queenless and queenright colony halves and monitored worker survival over a period of 60 days. Queen presence alone did not influence worker survival over the 60-day experimental period (χ^2= 0.0379, p= 0.538; Fig. 1A). However, worker survival differed significantly among sub-colony size categories (χ^2= 9.4, p= 0.009; Supplementary Figure S1). Pairwise comparisons averaged over queen presence revealed that workers from medium-sized sub-colonies exhibited the lowest mortality risk, surviving significantly better than workers from large sub-colonies (z = −6.99, BH-adjusted p < 0.0001) and small sub-colonies (z = −4.18, BH-adjusted p < 0.0001). In contrast, worker survival did not differ between large and small sub-colonies (z = 0.62, BH-adjusted p = 0.53). The interaction between queen presence and colony size was not statistically significant (χ^2= 5.36, p = 0.069). Nevertheless, simple-effect contrasts indicated that queen presence was associated with different mortality risks within sub-colony size categories: within large sub-colonies, workers in queenless colonies survived better than workers in queenright colonies (queenless vs. queenright: z = -4.182, BH-adjusted p < 0.0001), whereas in medium and small sub-colonies queen absence was associated with higher mortality (medium sub-colonies queenless vs. queenright: z = 2.381, BH-adjusted p = 0.017; small sub-colonies queenless vs. queenright: z = 4.284, BH-adjusted p < 0.0001; Fig. 1B).

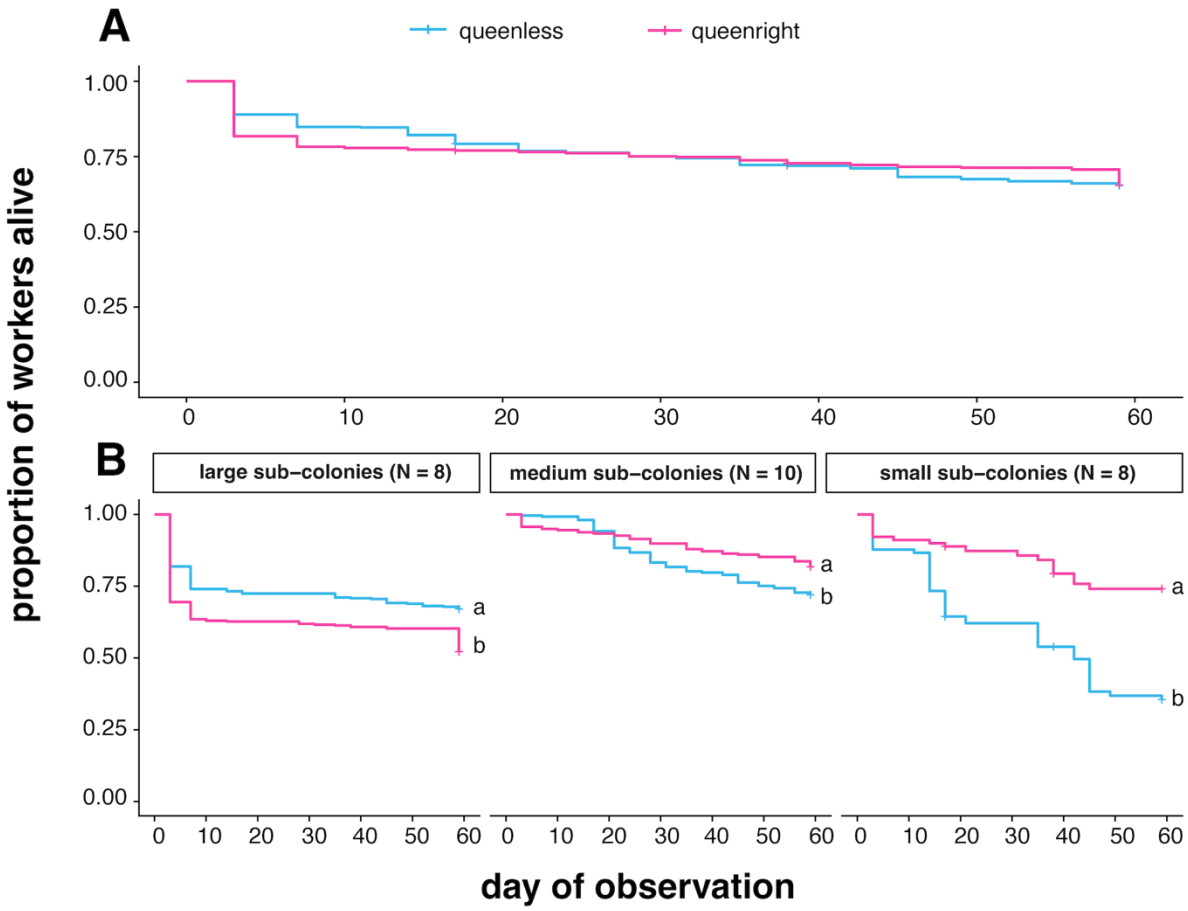

Figure 1: Queen presence and sub-colony size effects on worker survival in *Messor capitatus*.

A) Kaplan–Meier survival curves of workers in queenless (blue) and queenright (pink) sub-colonies. Queen loss alone did not influence worker survival over 60 days (χ^2= 0.0379, p= 0.538).

B) Kaplan–Meier survival curves of workers in queenless and queenright sub-colonies separated by sub-colony size category (large, medium, small). Numbers in facet titles indicate the number of sub-colonies per size category. Although the interaction between queen presence and sub-colony size was not statistically significant (χ^2= 5.36, p= 0.069), pairwise comparisons revealed colony size-dependent effects of queen presence on worker survival. In large sub-colonies, workers in queenless sub-colonies survived better than workers in queenright sub-colonies (queenless vs. queenright: z = −4.182, BH-adjusted p < 0.0001). In contrast, the direction of this effect differed in medium and small sub-colonies. Queen absence was associated with increased mortality (medium: z = 2.381, BH-adjusted p = 0.017; small: z = 4.284, BH-adjusted p < 0.0001). Letters indicate statistically significant differences between queenless and queenright treatments within sub-colony size categories.

Queen absence increased ovarian and egg development in workers

To examine whether queen presence influenced worker ovarian development, we analysed the ovarian profiles of workers from queenless and queenright colonies. We further tested whether worker behavioural caste (*i.e.,* worker location) as well as worker size influenced worker fecundity by comparing ovarian development not only between queenless and queenright colonies but also dependent on worker location and size.

We show that workers from queenless colonies had longer ovarioles (χ^2= 4.246, p = 0.039) and more oocytes in development (χ^2= 6.483, p = 0.011; Fig. 2A) than workers from queenright colonies. Moreover, we found that inside workers had similarly long ovarioles as outside workers (χ^2= 2.77, p = 0.096), while inside workers had more oocytes in development than outside workers (χ^2= 4.529, p = 0.033; Fig. 2B). There was no interaction between queen presence and worker location (mean ovariole length: χ^2= 1.493, p = 0.222; number of oocytes: : χ^2= 1.991, p = 0.158).

Additionally, major workers had longer ovarioles than medium workers (χ^2= 25.985, p < 0.0001), although the number of oocytes did not differ between the two worker morphs (χ^2= 2.456, p = 0.117). We found no interaction between worker location and body size regarding mean ovariole length (χ^2= 0.282, p = 0.595) and the number of oocytes (χ^2= 0.0084, p = 0.927). Sub-colony size did not show an overall effect on ovariole length (χ^2 = 4.175, p = 0.124) or on the number of oocytes (χ^2 = 3.480, p = 0.176). However, colony size interacted with worker body size for ovariole length (χ^2 = 7.239, p = 0.027). In large sub-colonies, major workers exhibited longer ovarioles than medium-sized workers (t = 5.12, BH-adjusted p < 0.0001), whereas this size-related difference was reduced or absent in small and (t = 1.87, BH-adjusted p = 0.07) medium sub-colonies (t = 2.0, BH-adjusted p = 0.05). Thus, the effect of worker body size on ovariole length became more pronounced with increasing sub-colony size (Supplementary Fig. S2). For the number of oocytes, caste-specific patterns (worker location) depended on colony size (χ^2 = 7.725, p = 0.021). While inside workers generally tended to have more oocytes in development than outside workers, this difference was most evident in medium sub-colonies (t = 3.25, BH-adjusted p = 0.002) and absent in large (t = -0.64, BH-adjusted p = 0.53) and small (t = 0.99, BH-adjusted p = 0.37) sub-colonies (Supplementary Fig. S3).

During the experimental period of 60 days, workers in five out of 13 queenless colony halves produced eggs (38.46%). Worker egg-laying occurred on days 17, 28, 35, and 52 post-queen removal, and only in one colony these eggs developed into larvae (Supplementary Table S2). None of the eggs produced by workers developed further into pupae or emerged as workers. At the end of the experiment, no worker-laid eggs were present in the colonies.

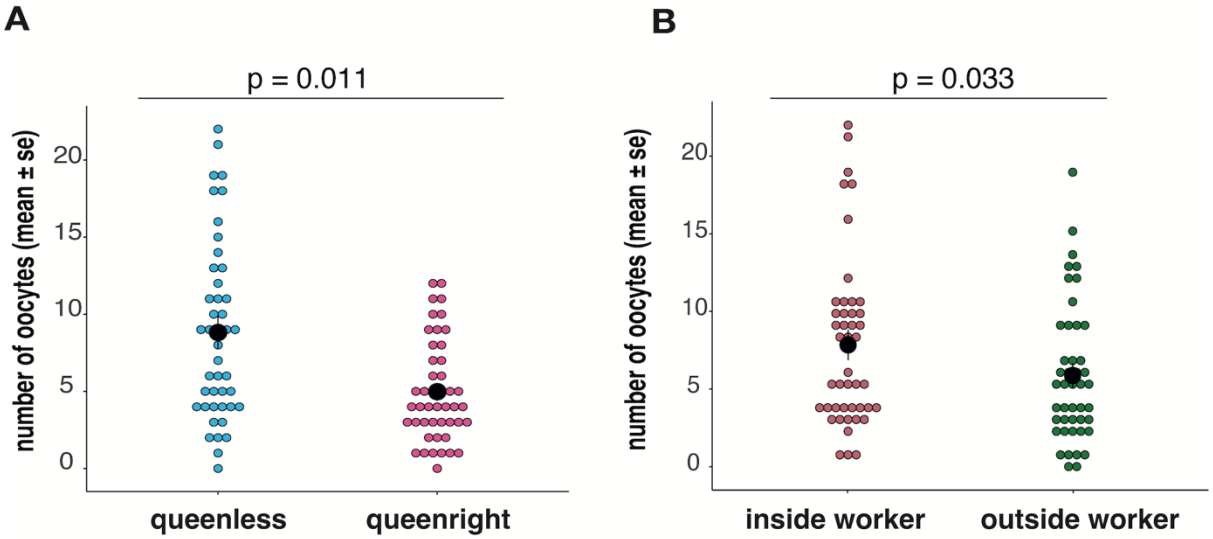

Figure 2: The influence of queen presence and worker location on ovarian and egg development in *Messor capitatus* workers.

A) Queen loss influenced ovarian and egg development as workers in queenless colonies (blue) had more oocytes in development than workers in queenright (pink) colonies (χ^2= 6.483, p = 0.011). **B)** Inside workers (pale red) had more oocytes in development than outside (green) workers (χ^2= 4.529, p = 0.033).

Discussion

The influence of queen loss on worker survival and ovarian development

Our study explored the influence of queen loss on worker survival and reproduction in the monogynous ant *Messor capitatus*. Workers of this ant species are reportedly capable to lay diploid offspring via thelytokous parthenogenesis (Grasso et al., 2000) in the absence of the queen. As worker reproduction is often associated with increased worker lifespan in many social insect species (Kohlmeier et al., 2017; Korb et al., 2021; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021b), we

aimed to find out whether queen loss would lead to increased worker survival and reproductive activity. Over an experimental period of 60 days, we monitored worker survival in queenright and queenless colonies and gauged worker reproduction as well as ovarian development dependent to queen presence.

Queen loss often induces pronounced changes in worker survival, physiology, immunity, and gene expression (Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021b). In several ant species, queen removal leads to increased worker lifespan and oxidative stress resistance, frequently accompanied by elevated reproductive activity (Kohlmeier et al., 2017; Majoe et al., 2021). We thus predicted that if workers of *M. capitatus* are indeed able to reproduce via thelytoky, queen loss would likely increase worker survival and that workers would switch to active reproduction and exhibit increased ovarian and egg development. Contrary to our expectations, queen presence alone did not influence worker survival over the 60-day experimental period, as workers in queenless sub-colonies survived equally well as those in queenright sub-colonies. However, worker survival differed among sub-colony size categories, with medium-sized sub-colonies exhibiting the lowest mortality, whereas survival did not differ between large and small sub-colonies. These findings indicate that sub-colony size is a major determinant of worker survival in *M. capitatus*, potentially reflecting differences in social buffering, task allocation, or physiological condition associated with group size.

Although the interaction between queen presence and sub-colony size was not statistically significant, post-hoc analyses revealed that the effect of queen loss on worker survival depended on sub-colony size. In large sub-colonies, queenless workers survived better than queenright workers, whereas in medium and small sub-colonies queen absence was associated with increased mortality. This size-dependent pattern suggests that the consequences of queen loss are context-dependent and may be mediated by the social and demographic structure of the colony. Even though the queen in social insects societies is of crucial interest and the loss of such important individuals can have far-reaching effects on the whole colony (Brunner and Heinze, 2009; Kronauer, 2009; Stroeymeyt et al., 2007), queen loss in larger groups may be partially buffered by increased worker numbers or social stability, whereas in smaller sub-colonies queen loss may represent a stronger stressor, potentially exacerbating mortality.

Despite the absence of a general queen effect on survival, queen loss strongly influenced worker ovarian development, as workers from queenless sub-colonies exhibited longer ovarioles and had more oocytes in development than workers from queenright sub-colonies. In social insects, fertile individuals often invest more in somatic maintenance and repair mechanisms (Negroni et al., 2020; Negroni et al., 2021b), and ovarian activation under queenless conditions may thus mitigate physiological stress. This interpretation aligns with the hypothesis that queen pheromones suppress worker reproduction and that their absence allows workers to activate their ovaries (Dijkstra and Boomsma, 2007; Stroeymeyt et al., 2007). The lack of an interaction between queen presence and worker location regarding ovarian development further suggests that ovarian activation occurred broadly across worker castes, rather than being restricted to specific behavioural groups (Konrad et al., 2012).

Although ovarian development increased in queenless sub-colonies, only a subset of orphaned colonies initiated egg production, and none of the worker-laid eggs developed into adult workers. This indicates that while queen absence triggers early stages of reproductive activation, successful reproduction may be constrained by additional factors not replicated in our experimental set-up. For instance, Grasso et al. (2000) monitored worker reproduction for more than ten months in larger, field-collected colonies, whereas our laboratory-reared colonies were smaller and observed for a shorter period. Since orphaned workers from larger sub-colonies in our study also exhibited higher survival, it is possible that the effects of queen loss on survival were outweighed or obscured by strong colony-size effects. The lack of overall survival differences could be attributed the duration of our experiment. We monitored our experimental colonies for 60 days, while Kohlmeier et al. (2017) monitored the workers for 200 days and found strong effects on worker survival based on queen presence. Thus, colony size, environmental conditions, and experimental duration likely interact to determine whether worker reproduction translates into increased survival.

Further, we found no clear evidence for successful thelytokous worker reproduction, as none of the worker-laid eggs developed into adult workers. This discrepancy with earlier findings (Grasso et al., 2000) could be attributed to several factors, including variations in maintenance conditions or the possibility that the ability

to produce female offspring by thelytoky is less widespread in this species than previously assumed. It is possible that only workers of certain populations possess the ability to produce female offspring. In the neotropical ant *Platythyrea punctata* reproduction predominantely occurs via thelyokous parthenogenesis while there are some populations that reproduce sexually with queens and males (Schilder et al., 1999). Moreover, in *Cataglyphis cursor* thelytokous parthenogensis plays an important role to counter high queen mortality so that workers could replace the queen when she dies (Lenoir et al., 1988; Pearcy et al., 2006). The queens used in our experiment originated from Spain, whereas previous reports stem from Italian populations, raising the possibility that thelytoky may be more prevalent in populations experiencing higher queen mortality.

Caste-and size-specific ovarian development

Our analyses further revealed that worker ovarian development varied with behavioural caste and body size. Inside workers, which are typically younger and less exposed to external risks, had more oocytes in development than outside workers, supporting the role of age polyethism in shaping reproductive potential (Bourke, 1988; Giraldo and Traniello, 2014; Kohlmeier et al., 2017; Lenhart et al., 2025; Majoe et al., 2021). This finding aligns with previous studies where younger, brood-caring workers (*i.e.,* nurses) are more likely to develop their ovaries in the absence of a queen (Kohlmeier et al., 2017; Lenhart et al., 2025; Majoe et al., 2021; Negroni et al., 2021b; Seistrup et al., 2023). Future studies might test whether increased ovarian development based on worker caste also leads to caste-specific differences in survival and oxidative stress resistance in *M. capitatus* (Kohlmeier et al., 2017; Lenhart et al., 2025; Majoe et al., 2021). Additionally, these caste-dependent differences in ovarian development interacted with colony size, indicating altered task allocation and demographic constraints that may influence both survival and reproductive physiology.

We also found that major workers had longer ovarioles than medium-sized workers, which contradicts our expectation that major workers are typically more specialised in tasks such as nest defence and food processing rather than reproduction

(Pie and Tschá, 2013). Indeed, a previous study investigating foraging behaviour in this species suggested medium-sized workers to be more likely involved in foraging activities (Grasso et al., 1998). Indeed, the difference in ovarian development was associated with sub-colony size, indicating that major workers are more likely to invest into reproductive physiology in larger colonies. However, the lack of a significant difference in the number of developing oocytes between the two worker morphs suggests that while larger workers may have the physiological potential for reproduction, they may not be more fertile in practice. Future studies might investigate worker task allocation dependent to the different worker sizes more closely as we did not find an interaction between worker behavioural caste and size. Thus, *M. capitatus* workers might engage in various task not only dependent to worker caste but also to size.

Conclusion

Our study contributes to the growing body of evidence that queen loss in social insects can stimulate reproductive activity in workers. However, in contrast to studies on other ants (Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021b) this does not universally increase worker survival in *Messor capitatus*. Instead, worker survival is strongly influenced by sub-colony size, and the effects of queen absence on survival depend on the social context in which workers operate. Medium-sized sub-colonies exhibited the lowest mortality overall, while queen loss had contrasting effects on worker survival across different sub-colony sizes.

Although we did not find clear evidence for successful thelytokous worker reproduction, the pronounced ovarian activation observed in queenless workers indicates a physiological readiness for reproduction that may become consequential under different ecological or demographic circumstances. Together, our findings highlight the importance of considering colony size and social context when assessing the consequences of queen loss in social insects and underscore that the relationship between reproduction and lifespan is not universal but shaped by colony-level factors. Further research integrating longer time scales, larger colonies, and population

comparisons will be essential to fully understand the role of worker reproduction in the life-history strategies of *M. capitatus*.

Author contributions

AL, BT and SF designed the experiment. BT conducted the experiments. AL analysed the data. AL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval. SF and AL supervised the study.

Funding

Funding came from the German Research Foundation (DFG grant nos. NE1969/4-1, FO 298/26-1, LI 3051/3-1) within the research unit FOR-2281 and for the GRK GenEvo 2526/1 – Project No. 407023052.

Acknowledgements

We thank Ina Knuf and Megha Majoe for their crucial support in raising the colonies as well as Romain Libbrecht for his helpful comments on the manuscript.

Data accessibility statement

The code and input data for all analyses are provided as electronic Supplemental Information.

Chapter 3

Supplementary Material

The impact of queen loss on worker survival and reproduction in the ant *Messor capitatus*

Anna Lenhart, Bianca Todorovic
and Susanne Foitzik

Unpublished manuscript

Chapter 3

Supplementary Figures

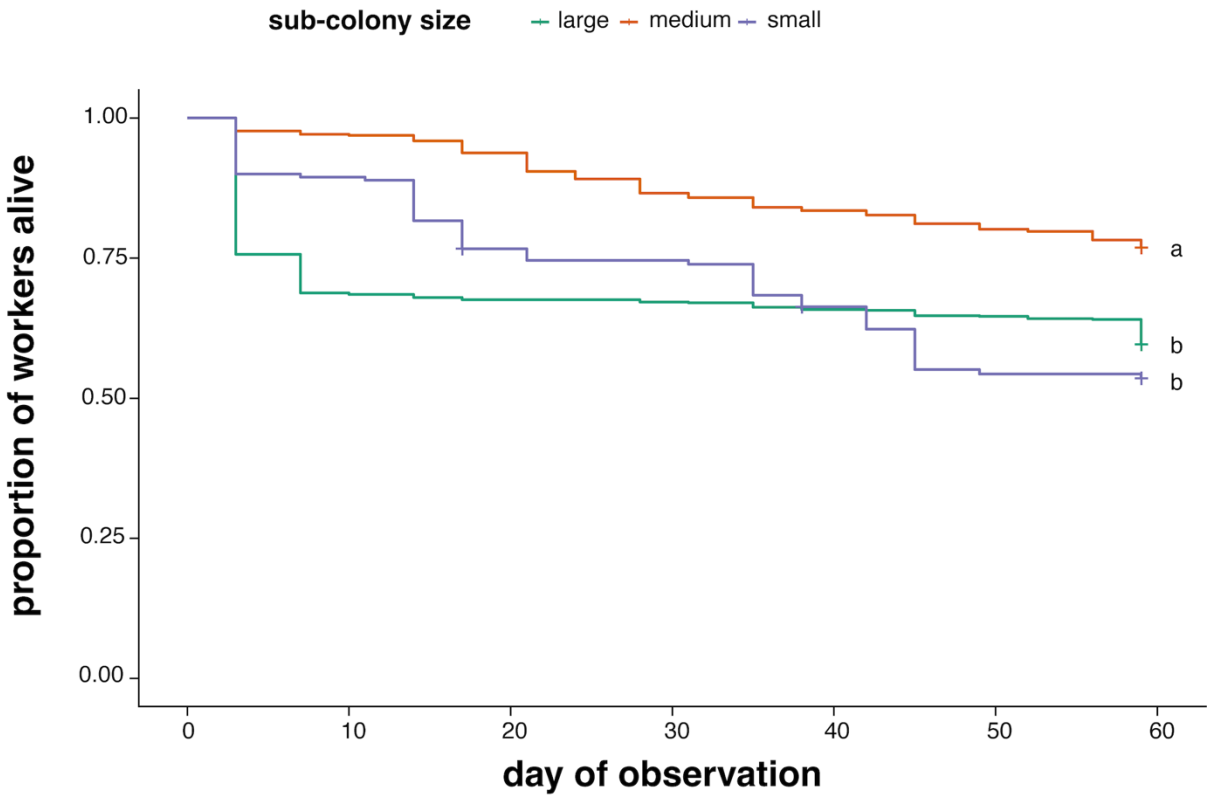

Figure S 1: Effect of sub-colony size on worker survival in *Messor capitatus*.

Kaplan–Meier survival curves of workers from large, medium, and small sub-colonies over a 60-day observation period, averaged across queenless and queenright treatments. Worker survival differed significantly among sub-colony size categories ($\chi^2 = 9.40$, $p = 0.009$). Pairwise revealed that workers from medium-sized sub-colonies (orange) exhibited the lowest mortality risk, surviving longer than workers from large (green) sub-colonies ($z = -6.99$, BH-adjusted $p < 0.0001$) and small (violet) sub-colonies ($z = -4.18$, BH-adjusted $p < 0.0001$). In contrast, worker survival did not differ between large and small sub-colonies ($z = 0.62$, BH-adjusted $p = 0.53$).

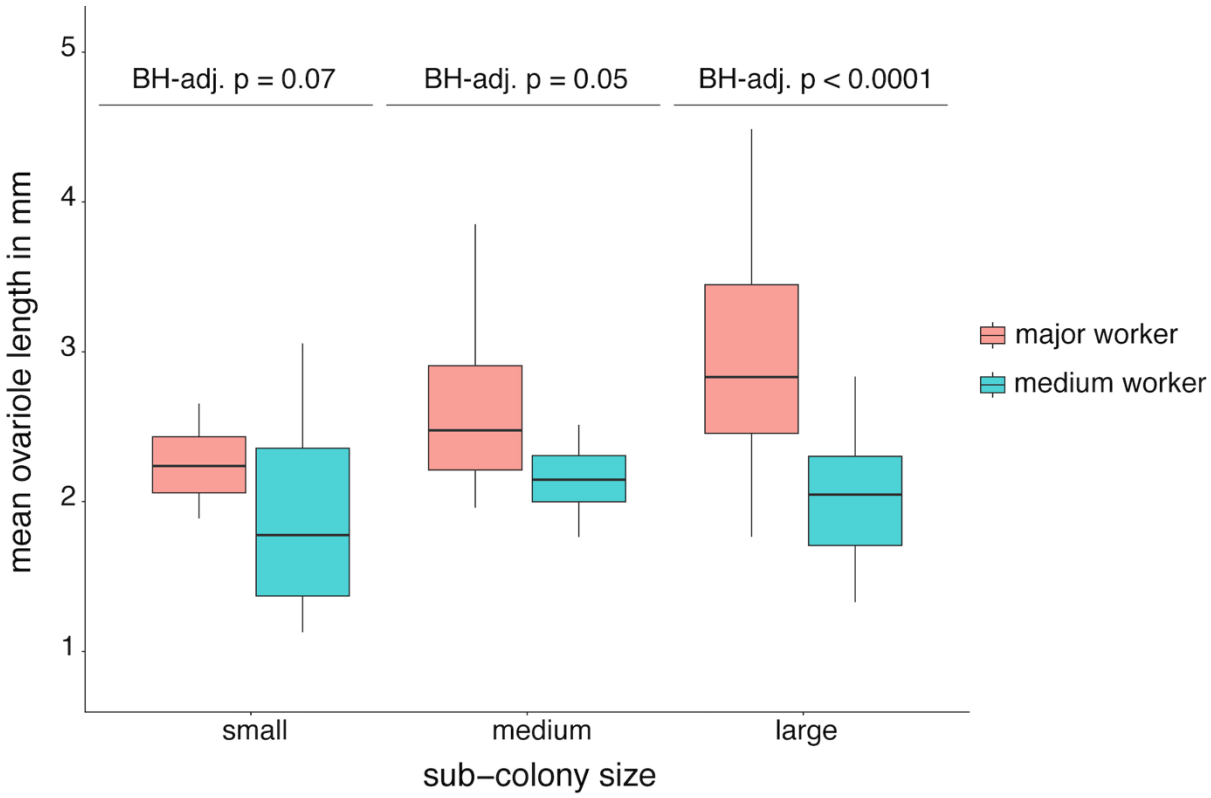

Figure S 2: Colony size and worker size dependent patterns in ovarian development

Mean ovariole length (mm) of major (red) and medium (blue) workers across small, medium and large sub-colonies. The difference in ovariole length between major and medium workers increased with sub-colony size and was significant in large sub-colonies ($t = 5.12$, BH-adjusted $p < 0.0001$), marginal in medium sub-colonies ($t = 2.0$, BH-adjusted $p = 0.05$), and not significant in small sub-colonies ($t = 1.87$, BH-adjusted $p = 0.07$). Boxplots show the median (horizontal line), interquartile range (box), and interquartile range (whiskers).

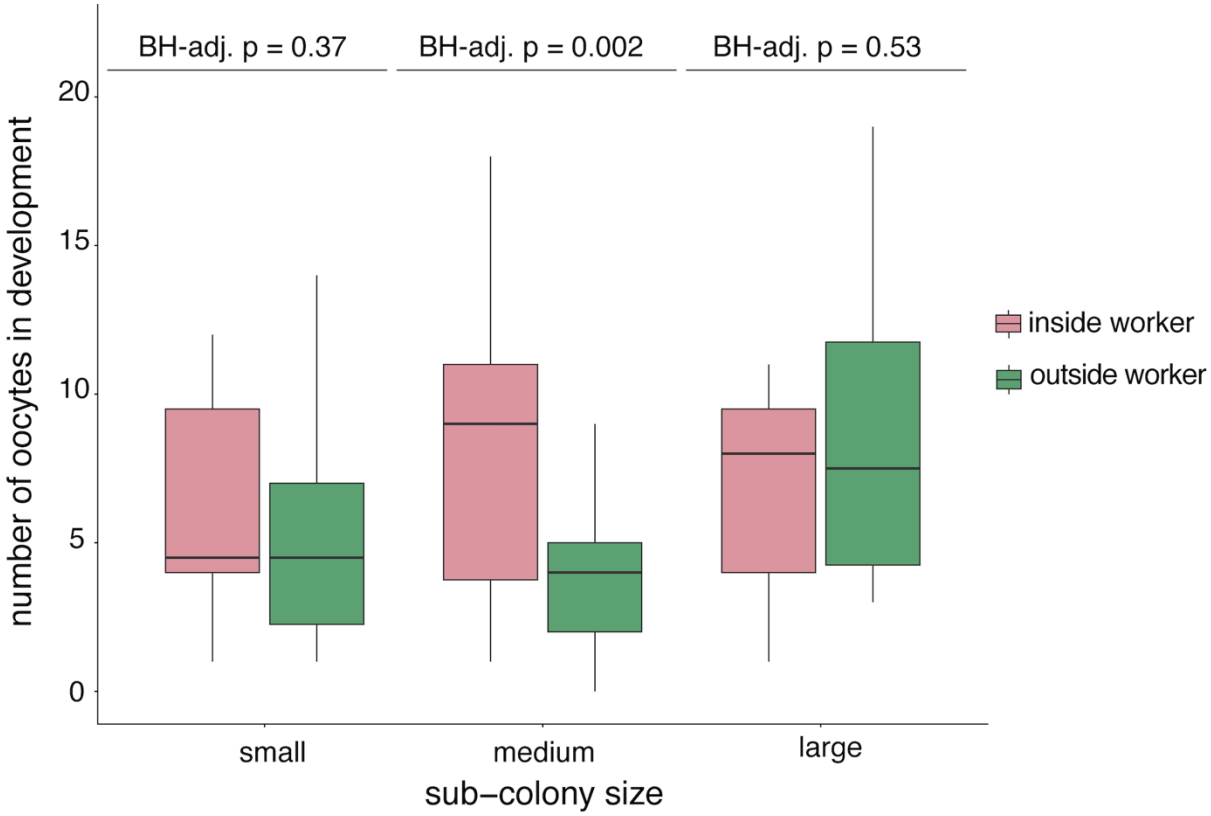

Figure S 3: Colony size and caste-dependent patterns in oocyte development.

Number of oocytes in development in inside (pale red) and outside (green) workers across small, medium, and large sub-colonies. Caste-related differences in the number of oocytes were most pronounced in medium sub-colonies (t = 3.25, BH-adjusted p = 0.002). This difference was absent in both small (t = 0.99, BH-adjusted p = 0.37) and large sub-colonies (t = -0.64, BH-adjusted p = 0.53). Boxplots show the median (horizontal line), interquartile range (box), and interquartile range (whiskers).

Chapter 3

Supplementary Tables

Table S 1: Composition and size classification of experimental sub-colonies used in the survival experiment.

The table lists cohort number, colony identity, number of queens (queenright = 1, queenless = 0), total number of workers per sub-colony at the start of the experiment, and the resulting sub-colony size category (small, medium, large). Each source colony was split into a queenright (A) and a queenless (B) sub-colony with equal worker numbers.

Cohort	Colony	Number of queens	Total number of workers	Sub-colony size category
1	MC7_A	1	48	medium
1	MC7_B	0	48	medium
1	MC23_A	1	26	small
1	MC23_B	0	26	small
1	MC43_A	1	70	large
1	MC43_B	0	70	large
1	MC51_A	1	85	large
1	MC51_B	0	85	large
1	MC52_A	1	49	medium
1	MC52_B	0	49	medium
2	MC24_A	1	55	medium
2	MC24_B	0	55	medium
2	MC30_A	1	62	medium
2	MC30_B	0	62	medium
2	MC39_A	1	100	large
2	MC39_B	0	100	large
2	MC40_A	1	43	medium
2	MC40_B	0	43	medium
2	MC53_A	1	115	large
2	MC53_B	0	115	large
3	MC28_A	1	26	small
3	MC28_B	0	26	small
3	MC38_A	1	14	small
3	MC38_B	0	14	small
3	MC45_A	1	24	small
3	MC45_B	0	24	small

Table S 2: Occurrence and development of worker-laid eggs in queenless sub-colonies of *Messor capitatus*.

Cohort	Colony	Date eggs observed	Days after queen removal	Development into larvae	Sub-colony size
1	MC7B	30.11.2023	17	yes	medium
1	MC23B	18.12.2023	35	no	small
2	MC24B	07.12.2023	17	no	medium
2	MC30B	18.12.2023	28	no	medium
1	MC43B	04.01.2024	52	no	large

Chapter 4

No lifespan extension in sterile workers of the neotropical ant *Hypoponera opacior* after queen loss

Anna Lenhart and Susanne Foitzik

Unpublished manuscript

Abstract

Obligate reproductive division of labour is a defining feature of social insect societies, where queens are extremely long-lived and highly fertile, while workers remain non-reproductive and live shorter lives. After queen loss, many facultatively sterile workers can activate their ovaries and extend their lifespans, highlighting a positive association between reproductive pathways and lifespan. However, this phenomenon has not been investigated in species with obligately sterile workers lacking any reproductive potential, leaving open the question of whether reproductive capability itself is essential for prolonging lifespan following queen loss. This study investigates the effect of queen presence on the survival of completely sterile workers in the ant *Hypoponera opacior*, a Ponerine species known for its unique reproductive system, which includes both gynomorphic and apterous worker-like queens with high levels of inbreeding. We created queenless and queenright colony halves and monitored worker survival over 42 days. Unlike other social insects with a more flexible reproductive ability, we found no evidence for a differential survival between queenless and queenright colonies. The complete sterility of *H. opacior* workers may explain why queenless colonies show no survival benefit: unlike in other social insects, where reproduction and longevity are positively linked, these workers lack the ability to activate reproductive pathways that could extend lifespan.

Keywords: social insects, reproductive division of labour, worker sterility, queen loss, lifespan, longevity/fecundity trade-off

Chapter 4

Introduction

One of the major evolutionary transitions is the shift from a solitary life to group-living (Bourke, 2011; Szathmáry and Smith, 1995). Among the factors influencing the evolution of sociality are increased reproductive success, enhanced foraging efficiency and reduced predation risk (Alexander, 1974; Clutton-Brock, 2002; Hamilton, 1964). In fact, group-living species can be found in almost all animal taxa and show a strong diversity of social organisations, from simple mated pairs to complex communities of individuals performing specialised tasks and complex societies with obligate reproductive division of labour (Costa, 2006; Wilson, 1971). For instance, social insects, including ants, termites, social bees and wasps, display significant caste-specific variations in lifespans and fecundities, largely driven by their highly specialised reproductive division of labour. In these species, reproduction is limited to queens (and kings in termites), while workers are responsible for tasks like brood care, foraging and nest defence and typically remain non-reproductive.

In most organisms, there is typically a trade-off between reproduction and longevity, with reproduction often shortening lifespan due to increased energy demands and heightened oxidative stress (Flatt, 2011; Kirkwood, 2017; Maklakov and Chapman, 2019; Mockett and Sohal, 2006). In social insects, this trade-off is reversed – queens are both highly fecund and long-lived, while most workers do not reproduce and live shorter lives (Keller and Genoud, 1997; Kramer et al., 2016). Although most workers cannot mate, many workers retain the ability of reproduction and are considered facultatively sterile as they are often able to produce haploid offspring (*i.e.,* males). This type of reproduction is defined as arrhenotokous parthenogenesis and typically only occurs in the absence of the queen (Crozier and Pamilo, 1996). This increased reproductive activity is reportedly associated with increased worker lifespans under queenless conditions (Kohlmeier et al., 2017; Kuszewska et al., 2017; Majoe et al., 2021). Further, the initiation of worker reproduction is often accompanied by behavioural and physiological shifts, such as increased oxidative stress resistance, decreased foraging activity, and changes in tissue-specific gene expression (Blacher et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021b). In these species, it is often the younger workers which typically remain inside the nest that are more likely to develop

their ovaries under queenless conditions compared to the workers that engage in foraging behaviour and are often older (*i.e.,* age polyethism) (Bourke, 1988; Blacher et al., 2017; Giraldo and Traniello, 2014; Seistrup et al., 2023; Tofilski, 2002).

Worker reproductive ability varies across species, especially between those with large, polygynous (multi-queen) colonies and species with monogynous (single- queen) colonies. In many large-colony species, worker sterility is the norm, particularly in invasive ants and many species with strong morphological caste differentiation (Aron et al., 2001; Korb and Heinze, 2016). Some species, however, possess totipotent workers that are capable of producing female offspring through thelytokous parthenogenesis and can even replace the queen (Rabeling and Kronauer, 2012). For instance, in the Ponerine ant *Platythyrea punctata*, dominant workers take over reproduction and live significantly longer than their non-reproductive subordinate sisters (Hartmann and Heinze, 2003). In *Harpegnathos saltator*, workers can mate and become gamergates (inseminated egg-laying workers), effectively replacing the queen and exhibiting extended lifespans compared to non-reproductive workers (Glastad et al., 2023; Opachaloemphan et al., 2021; Peeters, 1991). In various Ponerine species, worker ovaries and spermathecae resemble those of queens, enabling gamergates to take over the reproductive role in the colony (Peeters, 1993, 1991). In fact, in at least 100 of the 1,300 known Ponerine species, the queen caste is no longer produced during colony development (Peeters et al., 2000). Yet, high reproductive potential in ant workers is not universal. Some species rely on trophic egg-laying, which provide a nutrient-rich diet rather than reproductive offspring, possibly enhancing colony survival (Lenhart et al., 2025). Notably, extended worker lifespan, increased oxidative stress resistance, and gene expression changes have primarily been observed in species where workers can activate their ovaries for egg production, linking reproductive activity to lifespan (Blacher et al., 2017; Kohlmeier et al., 2017; Kuszewska et al., 2017; Majoe et al., 2021; Negroni et al., 2021b). To our knowledge, such patterns have not been observed in entirely sterile ant workers and raise the question whether the potential of ovarian activation is necessary for lifespan extension after queen loss, or whether obligately sterile workers without any reproductive potential rely on other mechanisms to maintain lifespan.

Here, we used the neotropical ant *Hypoponera opacior*, a Ponerine species characterised by its unusual presence of completely sterile workers (Foitzik et al., 2002), to explore the impact of queen loss on worker survival. *H. opacior* exhibits a remarkable reproductive system with both winged gynomorphic queens and apterous worker-like queens as well as winged and wingless males with distinct reproductive strategies, leading to the occurrence of monogynous and polygynous colonies (Foitzik et al., 2010, 2002). This reproductive diversity is accompanied with high levels of inbreeding, particularly in polygynous colonies with apterous worker-like queens (Foitzik et al., 2011b). Despite these elevated inbreeding levels, *H. opacio* does not exhibit signs of inbreeding depression, suggesting that this species has evolved mechanisms to cope with inbreeding while maintaining reproductive success (Kureck et al., 2012). Given that apterous worker-like queens are more frequently encountered than winged queens, we focused our experiments on colonies containing only worker-like queens (Foitzik et al., 2002). The presence of entirely sterile workers in this species provides the opportunity to explore how queen loss affects survival in a system where workers have no reproductive potential. Based on the lack of reproductive organs in *H. opacior* workers, we hypothesised that the incapability to activate their ovaries queen loss would decrease worker lifespan.

Material and Methods

Species collection

H. opacior colonies were collected in the Coronado National Forest (between 1,500 m - 1,880 m above sea level) in Southeastern Arizona in July 2022. Nests were found in the soil under rocks. After detection, all individuals and brood were collected with an aspirator and the soil was shifted to collect as much individuals as possible. Colonies were transferred to the close by laboratory at the Southwestern Research Station (31 °52.0000 N, 109 °12.6090 W) and the number of workers and brood items was counted. Each colony was kept in 50 ml Falcon tubes with a wire mesh in the lid to ensure air flow. The tubes were filled with soil and all individuals including brood were transferred to the tube. We kept the colonies at room temperature and fed them daily with mealworms or

frozen termite workers, dependent on the availability. Colonies were maintained under these conditions for no more than 20 days before they were transported to our laboratory at the Johannes Gutenberg University Mainz, Germany.

Laboratory maintenance

The colonies arrived at our laboratory end of July 2022 and were transferred into plastic boxes (15 cm x 13 cm x 5.5 cm) with an approximately 1.5 cm thick layer of soil on the ground. Since *H. opacior* colonies naturally build their nests under rocks, we placed a glass slide (5 cm x 8 cm) in the middle of each box, serving as rock equivalent, allowing us to observe the colonies underneath. The glass slides were covered with a piece of paper to ensure a dark environment for the ants. The colonies were kept at 21°C and were fed weekly with springtails and honey while the soil was always kept moist. Since *H. opacior* colonies were very sensitive to the laboratory environment and exhibited an overall high mortality, we kept the colonies at the most natural conditions as possible to increase colony survival under these conditions.

Queen removal experiment

Our aim was to investigate whether worker survival depended on queen presence. *H. opacior* exhibits alternative reproductive morphs of males and females associated with distinct sexual behaviours (Foitzik et al., 2010). Apterous worker-like queens are more commonly found than winged gynomorphic queens (Foitzik et al., 2002), which is why we decided to conduct our experiment with colonies that only contained worker-like queens. Although nests with worker-like queens are more common, the visual discrimination between worker and worker-like queen is challenging. We thus observed the colonies for two months prior our experiment and noted which nests produced eggs within this time frame. Since workers of *H. opacior* are sterile, we predicted that if egg-laying can be observed, the presence of at least one worker-like queen can be confirmed in the respective nest. We observed the presence of eggs in five of our colonies (mean number of workers 30.4 ± 10.53 sd). We then divided these five source colonies equally in two halves, assuming that one half would contain a queen (queenright treatment), and

the second half contains no queen (queenless treatment). Since we could not differentiate queens from workers beforehand, colony division was conducted blindly under the assumption that every source colony only contained one worker-like queen. If a colony had larvae present, the number of larvae was equally distributed to each colony half. None of the experimental colonies were provided with eggs. We monitored each colony half for 42 days and documented worker survival twice per week, while dead individuals as well as pupae were removed.

Ovary dissections

To confirm that our experimental colonies indeed contained either one or no queen, we dissected all individuals in each of the colony halves at the end of the monitoring period. All individuals that were alive at the end of the experiment, were flash frozen and dissected under a stereomicroscope (Leica S9i, Microsystems CMS GmbH, Wetzlar, Germany). We could confirm the presence of one worker-like queen in five of the 10 colony halves, while the other five colonies did not have a queen but only workers without any reproductive organs.

Statistical analyses

To investigate the effect of queen presence on worker survival, we used the *coxme* package version 2.2-18.1 (Therneau, 2024) in RStudio (R version 4.2.3, R Core Team, 2024) to build cox-regression mixed-effect models for the survival experiment. We included the treatment (queenless/ queenright) as fixed factor, and colony identity as random factor. Hypothesis testing was performed using the 'Anova' function from the package *car* version 3.1-2 (Fox et al., 2024). The package *ggplot2* version 3.4.2 (Wickham, 2016) was used to plot the Kaplan-Meier survival curves.

Results

No influence of queen presence on worker survival

To investigate whether the survival of sterile *H. opacior* workers is influenced by queen presence, we created queenless (N= 5) and queenright (N= 5) colony halves and monitored worker survival over a period of 42 days. The experimental colonies exhibited an overall survival of workers between 50% and 65% (Fig. 1). We found no evidence for an effect of queen presence on worker survival. In fact, workers in queenless colony halves died at similar rates as workers in queenright colony halves (χ^2= 1.78, p= 0.18; Fig. 1).

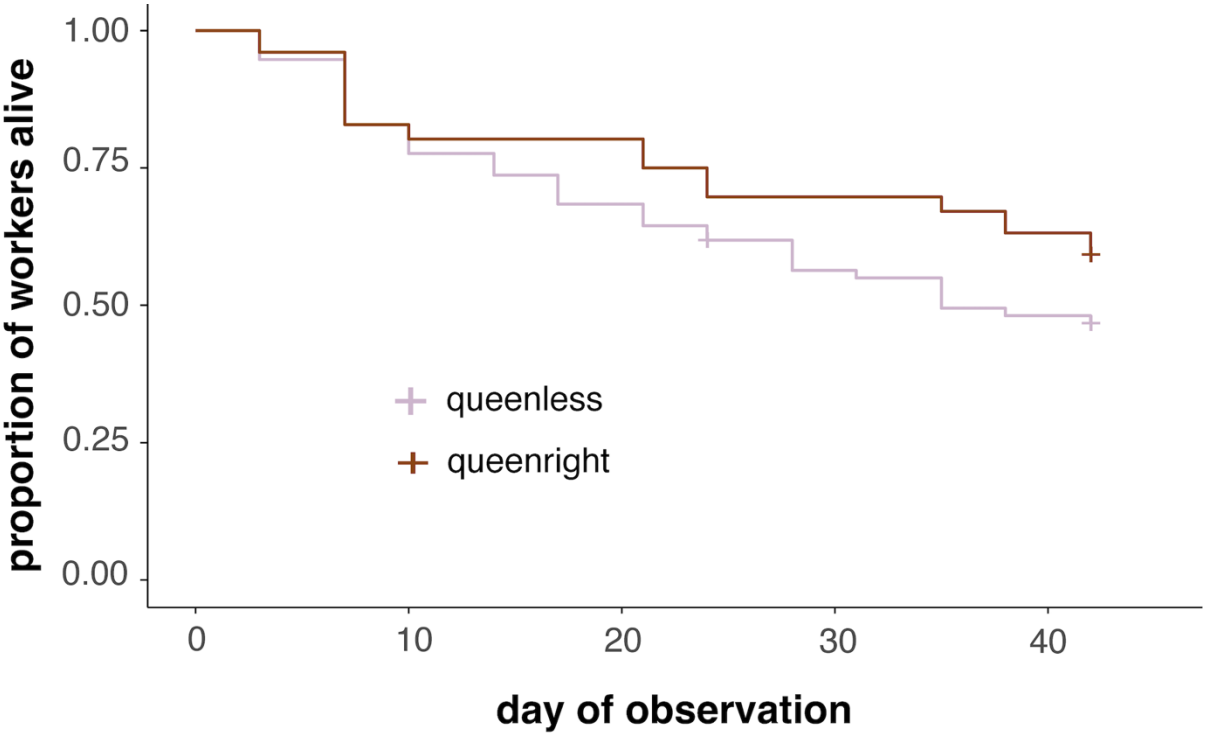

Figure 1: The influence of queen presence on worker survival over a period of 42 days. Queen loss did not affect worker survival. Queenless colonies (light violet line) exhibited a similar survival as queenright (brown line) colonies (χ^2= 1.78, p = 0.18).

Discussion

This study aims to explore whether queen loss can trigger survival benefits in species with obligately sterile workers that lack reproductive potential, addressing the question of whether reproductive capability is necessary for prolonging lifespan in the absence of the queen. We explored the influence of queen presence on worker survival in the ant *Hypoponera opacior*, a species with unique reproductive diversity, high levels of inbreeding and completely sterile workers (Foitzik et al., 2011b, 2010; Kureck et al., 2012). Our results revealed that queen presence did not significantly affect worker survival, as workers in both queenless and queenright colonies exhibited comparable mortality rates.

In many social insect species, queen loss triggers a shift in worker physiology, often leading to ovarian activation, increased stress resistance and the investment into antioxidant and immunity- related genes (Lenhart et al., 2025; Majoe et al., 2021; Negroni et al., 2021b). These physiological and molecular changes following increased ovarian activity and reproductive activity likely underlie the extended lifespans of orphaned social insect workers (Blacher et al., 2017; Kuszewska et al., 2017). In *H. opacior*, however, the irreversible sterility of workers eliminates the possibility to activate such pathways. Although our statistical analyses did not allow us to conclude differences in worker survival based on queen presence, there was a visual trend suggesting that workers in queenless colonies exhibited a slightly lower survival rate than those in queenright colonies (Fig. 1). Especially in species with obligately sterile workers, the loss of the queen ultimately results in colony demise if no further reproductive females can be produced from the brood. While the presence of the queen is often signalled by the release of specific pheromones (Breed and Gamboa, 1977; Holman, 2018; Holman et al., 2010), these signals cease after queen loss and leave the workers to tend the remaining brood until their own death. This behavioural adaptation can increase the worker's residual lifespan without the need to invest in direct reproduction. Consequently, workers in queenless colonies may maintain survival in order to tend the remaining brood, potentially explaining the absence of a pronounced decline in survival after queen removal in *H. opacior*.

However, since we did not find statistical evidence that the absence of reproductive potential decreased lifespan in sterile workers of this species, worker survival might be primarily shaped by environmental factors rather than internal physiological changes associated with reproductive status (Lopes et al., 2020). Sterility and its influence on lifespan have been explored in multiple biological systems, suggesting that group composition can influence survival even in non-insect societies (Zhu et al., 2023). This raises the question of whether social composition, rather than direct reproductive capability, could play a role in shaping the lifespan of sterile individuals across different taxa. Also, living in a group where the reproduction relies solely on one or two individuals, complete sterility may reduce the need for long-term survival strategies typically associated with reproductive potential.

An intriguing aspect of *H. opacior* is its reproductive system with winged gynomorphic queens and apterous worker-like queens (Foitzik et al., 2010). This diversity in queen morphs could play an important role in shaping both worker sterility and survival dynamics. Unlike species where queen removal induces reproductive flexibility in workers, *H. opacior* workers appear to have a more fixed, sterile state. The presence of these two distinct queen morphs may reduce the evolutionary need for workers to maintain reproductive potential, which in other species may act as an evolutionary adaptation in case of queen loss. Also, in *H. opacior* high levels of inbreeding have been observed, particularly in polygynous nests headed by worker-like queens (Foitzik et al., 2011b). While lower relatedness among individuals can reduce the likelihood of division of labour being strongly favoured (Cooper and West, 2018; Madgwick et al., 2018), the increased relatedness resulting from such high inbreeding may reinforce a more rigid division of labour. This could help explain the complete lack of reproductive potential in the worker caste. However, this does not appear to result in inbreeding depression (Kureck et al., 2012). The absence of inbreeding depression, coupled with the ability to regulate reproductive allocation towards the production of sexuals in more inbred colonies, suggests that mechanisms to cope with inbreeding evolved, while high reproductive output by the queens and obligate sterility in workers can be maintained. Additionally, since workers in inbred colonies are larger, this could also influence reproductive dynamics (Kureck et al., 2012). Larger workers might be more specialized

for non-reproductive tasks (Pie and Tschá, 2013). Thus, larger, more inbred workers may have other physiological adaptations that reduce the need for reproductive flexibility.

In the broader context of lifespan evolution, it has been proposed that longevity is not merely a consequence of reproductive capacity, but rather a complex interplay between metabolic processes, environmental conditions, and immune system investments (Gems and Partridge, 2013). It is possible that species-level factors, such as inbreeding tolerance and alternative reproductive strategies, have a stronger influence on worker longevity than reproductive status or queen presence alone. To advance our understanding of the molecular mechanisms that influence worker survival in sterile species like *H. opacior*, additional research is necessary. Investigating gene expression profiles with a focus on stress resistance, metabolic functions, and immune responses in sterile workers could shed light on the physiological factors that support worker lifespan in the absence of reproductive roles. Moreover, comparing survival patterns across additional ant species with obligately sterile workers may reveal broader trends in the evolution of eusociality, providing insights into how sterility and survival are regulated in diverse social insect systems.

Conclusion

Our study provides novel insights into the survival dynamics of obligately sterile workers in the ant *Hypoponera opacior* and shows that queen presence does not significantly influence worker lifespan. In contrast to other social insect species where reproductive flexibility in the absence of the queen affects lifespan (Blacher et al., 2017; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Seistrup et al., 2023), the irreversible sterility of *H. opacior* workers may have evolved as a response to the reproductive structure of their colonies, particularly the presence of distinct queen morphs. The high levels of inbreeding in *H. opacior*, especially in polygynous colonies, may further reinforce this highly specialized reproductive division of labour and contribute to irreversible sterility of the worker caste (Foitzik et al., 2011b; Kureck et al., 2012). Our findings suggest that factors beyond queen presence, such as inbreeding and environmental conditions, may play a pivotal role in shaping worker lifespan. Future

studies should investigate the molecular and physiological mechanisms driving worker survival, particularly in highly inbred species, to explore whether these findings represent a broader trend within social insects.

Author contributions

AL and SF designed the experiment. AL conducted the experiments. AL analysed the data. AL led the writing of the manuscript. SF contributed critically to the drafts and gave final approval. SF supervised the study.

Funding

Funding came from the German Research Foundation (DFG –GRK2526/1 –Project No. 407023052 and Project DFG FO 298/26-1.

Acknowledgements

We thank Juliane Hartke for her support in ant collection and Stefanie Emmling, as well as Marion Kever for their support in the laboratory. We also thank Romain Libbrecht for his helpful comments on the manuscript.

Data accessibility statement

The code and input data for all analyses are provided as electronic Supplemental Material.

Chapter 5

Absence of supergene-linked determination of wing polymorphism and alternative reproductive strategies in the ant *Hypoponera opacior*

Anna Lenhart, Hugo Darras, Susanne Foitzik

Accepted at: Biology Letters (as of February 2026)

Abstract

Social insects frequently exhibit intraspecific variation in reproductive strategies, characterised by pronounced differences in the morphology, physiology, and behaviour of reproductive individuals and the social organisation of the colony. In many ant species, this complex variation is governed by supergenes that control multiple traits, including queen morphology and dispersal behaviour, as well as the number of queens that can coexist within a colony. Here, we investigate the genetic basis of sexual dimorphism in the ant *Hypoponera opacior*, which produces both winged and wingless morphs in males and queens, each associated with distinct reproductive behaviours and seasonal activity. Using whole-genome resequencing of all morphs, we tested for signatures of supergene architecture. Our analyses revealed no evidence for a supergene or other large genomic rearrangement underlying this morphological and behavioural diversity. These findings suggest that male and queen morph determination in *H. opacior* is not controlled by a large structural polymorphism and may instead result from phenotypic plasticity, environmentally sensitive developmental switches, or polygenic factors. Our study demonstrates that complex social traits in ants can evolve and persist without supergene architectures and underscores the importance of considering alternative genetic and epigenetic mechanisms underlying reproductive polymorphisms.

Key words: supergenes, polymorphism, social insects, caste morphology, phenotypic plasticity

Chapter 5

Introduction

Phenotypic plasticity, the ability of a single genotype to give rise to distinct phenotypes in response to varying environmental conditions, is a key mechanism shaping developmental and life-history diversity (Moczek et al., 2011; Pigliucci, 2001; Schlichting and Pigliucci, 1998; West-Eberhard, 2003). It underlies a wide range of behavioural, morphological and physiological traits, enabling organisms to respond rapidly to changing environments. In many species, this flexibility allows a stable co-existence of individuals with discrete phenotypes within the same population.

In social Hymenoptera (ants, some bees and wasps), phenotypic plasticity plays a central role in the development of complex social structures. Phenotypic plasticity underlies much of the remarkable developmental diversity observed in social insects, enabling queens, workers, and males to diverge in morphology, physiology, and behaviour despite sharing a largely identical genome (Schwander et al., 2010; Simpson et al., 2011; West-Eberhard, 2003). Many ant species also exhibit pronounced morphological and behavioural variation within castes. Workers may differentiate into specialized morphs such as soldiers or replete specialized in food storage, while reproductive castes often display alternative morphologies linked to distinct reproductive tactics (Hölldobler and Wilson, 1990; Wilson, 1971). In several ant lineages, queens and males occur as winged or wingless (ergatoid) forms that differ in dispersal capacity, mating behaviour, and colony-founding strategy (Foitzik et al., 2010, 2002; Heinze and Tsuji, 1995). Understanding these dimorphisms helps explain complex social traits.

Although polymorphisms in ant reproductive strategies were long considered environmentally induced, it is now clear that alternative social structure and reproductive strategies are often underpinned by genetic architectures. Supergenes – extended, non-recombining genomic regions containing co-adapted loci (Gutiérrez-Valencia et al., 2021) – have been shown to regulate reproductive traits across the ant phylogeny, including in *Cataglyphis niger* (Lajmi et al., 2025), *Formica* spp (Brelsford et al., 2020; Lagunas-Robles et al., 2021; Scarparo et al., 2023), *Leptothorax acervorum* (Braim, 2015), *Myrmecina graminicola* (Mona et al., 2025), *Myrmica ruginodis* (Sigeman et al., 2025), *Ooceraea biroi* (Trible et al., 2023), *Pogonomyrmex californicus*

(Errbii et al., 2022), and *Solenopsis invicta* (Pracana et al., 2017; Purcell et al., 2014; Wang et al., 2013). These architectures facilitate the co-inheritance of allele combinations specifying traits such as the number of queens sharing reproduction within a nest, their dispersal strategy and morphology, as well as caste determination (Buschinger, 1978; Buschinger and Schreiber, 2002; Gutiérrez-Valencia et al., 2021; Heine and Buschinger, 1989; Mona et al., 2025; Purcell et al., 2014; Scarparo et al., 2023; Sigeman et al., 2025; Thompson and Jiggins, 2014; Wang et al., 2013). In many of these species, a clear genetically determined queen size dimorphism occurs, with smaller "microgyne" queens typically associated with multiple-queen societies and a loss of independent colony foundation potential (Scarparo et al., 2023; Sigeman et al., 2025). Moreover, in at least three ant species, namely *Harpagoxenus sublaevis*, *Leptothorax sp. A*, and *M. graminicola*, queens show wing polymorphisms that are determined by a single locus or a supergene (Buschinger, 1978; Buschinger and Schreiber, 2002; Heine and Buschinger, 1989; Mona et al., 2025), a phenomenon also observed in other insects, including stoneflies and male aphids (Li et al., 2020; Veale et al., 2018).

The ponerine ant *Hypoponera opacior* exhibits a wing polymorphism in both queens and males, each associated with distinct reproductive strategies and colony structures (Foitzik et al., 2002, 2011b). Winged sexuals (queens and males) disperse during early-season mating flights, following which, dealate mated queens typically establish monogynous (single-queen) colonies. These founding nests are common, comprising half of all nests headed by dealate queens. In contrast, ergatoid queens mate with ergatoid males within the nest later in the season and typically remain in their natal nest leading to highly inbred polygynous (multiple-queen) colonies (Foitzik et al., 2011a; Kureck et al., 2012) (Fig. 1A). Wingless queens also disperse, but do so with the help of worker sisters over short walking distances, leading to a viscous population structure among larger nests at a local scale (Foitzik et al., 2011b). Despite regular inbreeding (F_{is} = 0.43), within-colony relatedness in these nests is low ($r \approx 0.2$) (Foitzik et al., 2011b). Although most ergatoid males are produced late in the season, some emerge earlier and mate with winged nestmate queens before these eclose from their pupae.

So far, no association has been detected in *H. opacior* between the sexual morphs produced by colonies and the morph of the mother queen. However, the seasonal and demographic patterns displayed by each morph are compatible with a potential genetic basis for morph variation, as observed in other ants; assuming a single locus basis for queen and male morphologies, colonies headed by parents carrying different alleles could produce reproductive individuals of both morphs each year, particularly in polygynous nests. Given the prevalence of supergenes underlying reproductive and social polymorphisms in ants, and of a genetic basis for wing polymorphism in several insect taxa, we asked whether the large phenotypic differences observed between winged and ergatoid morphs of *H. opacior* are genetically controlled by a supergene polymorphism. We performed whole-genome resequencing of individuals representing each morph and analysed genetic differentiation, linkage disequilibrium patterns, relative read depth, and SNP–trait associations. Our results reveal no evidence for a supergene or large structural variation determining reproductive morph in *H. opacior*, indicating that complex alternative reproductive strategies can evolve in the absence of supergenes, despite the growing number of recent studies linking such polymorphisms to supergenes in other taxa.

Material and Methods

Species collection

H. opacior colonies were collected under rocks (between 1,500m –1,880m above sea level) in the Coronado National Forest in southeastern Arizona, within a 5km radius (collection permit OMB NO. 0596-0082), in July 2022. Entire colonies (sexuals, workers and brood) were sampled when possible. We maximised sample independence by collecting individuals from as many colonies as possible and by sampling distinct colonies spaced at least 2m apart. Winged and ergatoid males, as well as winged queens were directly conserved in 0.8-1.5 ml DNA/RNA Shield (Zymo Research). As ergatoid queens require behavioural observations and ovary dissections for identification (Foitzik

et al., 2002), colonies were transported to our laboratory (Mainz, Germany) in 50ml Falcon tubes containing soil and a moist cotton ball, with a wire-mesh lid for ventilation.

Identification of ergatoid queens

Colonies were maintained in plastic boxes (15 × 13 × 5.5 cm) with ~1.5 cm soil and a 5 × 8 cm glass slide serving as a rock analogue, enabling observation beneath the nest. Slides were covered with paper to maintain darkness. The colonies were kept at 21 °C, fed weekly with springtails and honey, and the soil was kept moist. Winged sexuals emerging in the laboratory were preserved in DNA/RNA Shield as described above. Colonies were monitored for two months for egg production to detect reproductive ergatoid queen(s), since sterile workers of *H. opacior* do not possess ovaries. Five egg-laying colonies were frozen at −80 °C and all females were subsequently dissected in 1× PBS under a Leica S9i stereomicroscope. Individuals with developed ovaries were classified as ergatoid queens and used for genetic analyses. We identified five ergatoid queens.

DNA extraction and sequencing

For DNA extraction, we selected 16 males from 16 different colonies. Of these, five were winged and 11 were ergatoid. Additionally, we sampled 16 queens, each from a separate colony. Of these, 11 were winged or dealate queens, and five were ergatoid. Four colonies contributed both a male and a queen, resulting in DNA samples of 32 individuals from 29 colonies (Table S1). Of these, we sampled four ergatoid males, one winged male, and one dealate queen from our laboratory colonies in Germany (Table S1). All samples were preserved in DNA/RNA Shield under the same conditions until DNA extraction.

The ants were homogenized with a Percellys Evolution Touch (5800 RPM, six cycles with 30s pause at 4°C) and DNA was extracted using a DNA/RNA Microprep Kit Protocol (Zymo Research). Sequencing (Illumina NovaSeq X Plus Series, PE 150bp). Whole genome sequencing was performed by Novogene (Cambridge, UK). We obtained an average sequencing depth of 51.18× across all 32 samples, with a standard deviation

of 17.03×, based on a reference genome size of approximately 250.8 Mb. This coverage ensured high confidence in variant detection and downstream analyses.

Variant Calling and Filtering

Sequence alignment was performed using the high quality GAGA reference genome (GCA_048543595.1), in which 95 percent of the assembly is contained within 37 scaffolds (Vizueta et al., 2025). This genome was generated from stLFR and Illumina whole-genome sequencing of a worker, a larva, and an ergatoid male (SRR31149436, SRR31046815, SRR31046816, SRR31046817). We aligned sequences using BWA136 v0.7.17 (Li and Durbin, 2009) and variant calling was performed simultaneously on males and females using FreeBayes v1.3.6 (Garrison and Marth, 2012), treating haploid males as pseudo-diploids. Variants were filtered to retain high confidence polymorphisms. Indels were removed prior to analysis. Specifically, loci were retained if they were biallelic (--min-alleles 2, --max-alleles 2) with an alternative allele supported by at least three copies (--mac 3). In addition, we discarded loci with more than 50% missing data (--max-missing 0.5) or outlier coverage depth (--min-meanDP 18.51, --max-meanDP 74.02). Stricter filtering, allowing only 10% missing data had no significant impact on the final dataset, reducing the final SNP count by only 0.24%.

In Hymenoptera, female development requires heterozygosity at the multi-allelic sex determination locus (CSD), whereas males typically develop from unfertilized hemizygous eggs. However, regular inbreeding can result in some species, such as in *H. opacior,* in the occasional production of CSD-homozygous eggs that develop into non-functional diploid males (Kureck et al., 2013). To identify and exclude such unusual diploid males, we compared whole genome heterozygosity between males and females. This revealed one male sample (B22_wM) with heterozygosity similar to the diploid females, which was removed from the subsequent analyses (Fig. S1).

Genetic Differentiation and Association Analyses

Supergene regions are typically characterised by elevated genetic differentiation (e.g., high F_{ST} values), strong linkage disequilibrium, suppressed recombination, and distinct

genotype-phenotype associations (Gutiérrez-Valencia et al., 2021; Thompson and Jiggins, 2014). Genetic differentiation between the morphs was first assessed using whole-genome principal component analyses (PCA) performed with PLINK v1.9 (Purcell et al., 2007), and rolling PCA analyses in non-overlapping 50 kb windows with the R package SNPRelate considering only windows with at least 10 SNPs (Zheng et al., 2012). Next, we estimated genetic differentiation across their genomes using Weir and Cockerham's F_{ST} calculated in 20 kb sliding windows with no overlap using VCFtools v.0.1.16 (Danecek et al., 2011). In addition, a Genome-Wide Association Study (GWAS) was conducted in PLINK v1.9. using the --assoc function with the Fisher's exact test. Benjamini-Hochberg correction (FDR-adjusted $p \leq 0.05$) for multiple testing was applied subsequently in R and the results were visualized using the 'qqman' package v0.1.9 (Turner, 2018). For each GWAS comparison, genotypes at significant SNPs were extracted using the R package vcfR v.1.15.0 (Knaus and Grünwald, 2016). To determine whether significant SNPs mapped to annotated genes, their coordinates were intersected with the GAGA reference annotation using bedtools v2.29.2 (Quinlan and Hall, 2010). To rule out the possibility that the morphs are determined by a large insertion present only in one morph type, read coverage analyses were performed. Per-base read depth was calculated for each individual using bedtools v2.29.2 genomecov (Quinlan and Hall, 2010), and normalized by dividing each value by the most frequent coverage value observed across the genome. The normalized depth profiles were then compared across morphs to identify regions with potential morph-specific coverage depletion. In addition, we compared global mapping statistics among morphs using samtools flagstat and tested differences in mapping rates with the Wilcoxon rank-sum test.

Results

No genetic differentiation between morphs

To investigate whether winged and ergatoid reproductive morphs of *H. opacior* differ at the genomic level, we first tested whether the two morphs form distinct genetic clusters. Principal component analysis revealed no evidence of discrete clustering, as none of the

main principal components separating ergatoid and winged morphs either across sexes or within males and queens (Fig. 1B–C; Figures S2–S3). Consistent with these results, genome-wide genetic differentiation between ergatoid and winged morphs was virtually zero (average in 20kb window, F_{ST} =0.0051; Fig. 1D), and separate analyses of males and queens likewise revealed no differentiation among morphs (average in 20kb windows, F_{ST} = −0.014 and −0.007, respectively; Fig. S4A–B).

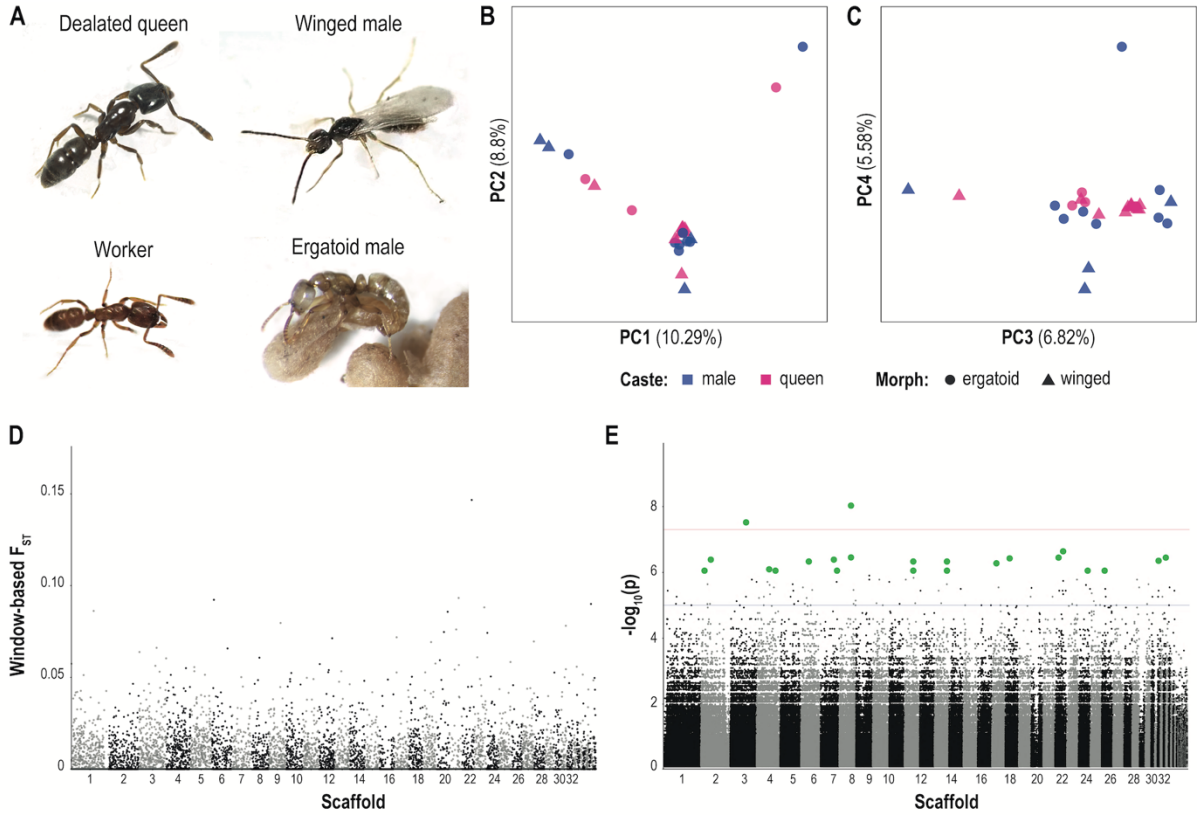

Figure 4: Phenotypic and genomic contrasts between ergatoid and winged *Hypoponera opacior* males and queens.

(A) *Hypoponera opacior* ants, clockwise: a dealate (formerly winged) queen, a winged male, an ergatoid male mating with a presumably ergatoid queen inside her cocoon, and a worker. Ergatoid queens closely resemble workers and are typically distinguishable only through ovary dissections (Foitzik et al., 2002). **(B)** Components of the principal component analysis (PCA) of ergatoid and winged males and queens: the first two principal components. **(C)** Principal components three and four. Morph is not associated with strong genetic variances. The percentage of variance explained by each axis is indicated. **(D)** Mean F_{ST} values (in 20 kb non-overlapping windows) between ergatoid and winged morphs across males and queens, plotted by scaffold and genomic position. Alternating black and grey points distinguish adjacent scaffolds. No scaffold shows a concentrated region of elevated differentiation, indicating an absence of a large non-recombining genomic region associated with wing polymorphism. Similarly, we did not detect any regions with elevated F_{ST} among morphs within males and queens separately (Fig. S8). **(E)** Manhattan plot of the GWAS between ergatoid and winged morphs in males and queens across scaffolds. Each point represents a single SNP, plotted by genomic position across scaffolds. Association significance is shown on the y-axis as $-\log_{10}$ (p-value). Green dots indicate SNPs significant after BH-correction. In total, 23 out of 1,054,360 SNPs were significant (BH-adjusted $p \leq 0.05$). The solid blue line denotes the conventional suggestive threshold ($p = 10^{-5}$) and the solid red line denotes the conventional genome-wide significance threshold ($p = 5 \times 10^{-8}$). Two of the significant SNPs exceed the genome-wide significance threshold**.** Photo credit: Susanne Foitzik and Megha Majoe.

No supergene controlling sexual morph

To further investigate whether the morph determination in *H. opacior* males and queens could be determined by a supergene, we investigated genomic differentiation across the genomes of ergatoid and winged morphs. Sliding-window PCA revealed only two regions of extended linkage disequilibrium where samples clustered, albeit not according to sex or morph (2 Mb on Scaffold 1 and 3 Mb on Scaffold 16; Figures S5–S8). Inspection of genotypes revealed that the two intervals are in perfect linkage disequilibrium. Because the reference genome is not resolved to chromosome level, these intervals likely represent a single underlying genomic region split between scaffolds. These intervals also show modestly elevated F_{ST} values relative to the genome-wide background (Fig. 1D), spanning several megabases; however, differentiation remains low and is not associated with morph or sex (Fig. 1D). However, in males, four small windows in the middle of four large (>2.7 Mb) scaffolds yielded elevated F_{ST} values among morphs (scaffolds 3, 20kb window with F_{ST} = 0.381; scaffold 17, 20kb window with F_{ST} = 0.364; scaffold 22, 20kb window with F_{ST} = 0.504; and scaffold 31,20kb window F_{ST} = 0.461; Fig. S4A), that we interpreted as dispersed, false positive signals. Similarly, genome-wide association testing did not reveal a concentrated signal on any specific scaffold. Furthermore, although 23 of 1,054,360 SNPs showed significant allele frequency differences between morphs (Fisher's exact test, all BH adjusted $p \leq 0.05$), including two loci surpassing the conventional genome-wide significance threshold (Scaffold 3, BP 7,528,889 and Scaffold 8, BP 5,629,530; both BH adjusted $p < 0.017$; Fig. 1E; Table S2), none exhibited perfect association with morph segregation, particularly in queens, where all three genotypes occurred in each morph (Table S3; Fig. S9). Separate analyses for each sex, revealed no SNP associated with morphs in queens (BH-adjusted p all > 0.05; Fig. S10B), but 166 significant SNPs in males (Fig. S10A). Of these, 55 SNPs showed complete genotype–phenotype association in males, and 41 mapped to 35 predicted genes (Table S4). However, these SNPs were dispersed across the genome (Fig. S11), and their number is consistent with what would be expected by chance given the sample size (exact permutation test: p = 0.327). Given our statistical power, we cannot exclude contributions from smaller-effect loci or sex-specific genetic variants. Nevertheless, our

results show that morph differentiation in *H. opacior* is not determined by a single large locus and does not share a common genetic basis across the sexes.

No morph-dependent coverage differences

Stable polymorphism can be encoded by large indels, as is the case for distyly in *Linum* plants (Gutiérrez-Valencia et al., 2022), which may be overlooked when assessing only genetic differentiation among morphs. To confirm that *H. opacior* morphs are not genetically determined by such polymorphism, we conducted a coverage analysis to compare read depth between morphs. We expected that if a large insertion determined one morph, it would show differences in coverage among morphs compared to genome-wide levels (e.g., half coverage if hemizygous or zero coverage if present in the genome assembly but absent from the sample). No region showed consistent morph-specific depletion in coverage (Wilcoxon rank-sum tests, Bonferroni-corrected p-values ≥ 1 for each of the 250,796,673 genomic positions; Fig. S12). Because a large insertion could be missing from the reference genome assembly, we also assessed whether one morph had more unmapped reads, suggesting an unassembled insertion. Mapping rates did not differ between morphs (Wilcoxon rank-sum test: $W = 68$, $p = 0.185$), with mean mapping rate 96.73 ± 9.28% SD. Only one sample (a winged male) had a lower mapping rate (46.48%; Table S5). These results further confirm that dimorphism in *H. opacior* is not genetically determined.

Discussion

To date, nearly all reported studies investigating the genetic basis of social organization or queen morphology in ants have identified a genetic component, and often supergenes (Braim, 2015; Brelsford et al., 2020; Kay et al., 2022; Lagunas-Robles et al., 2021; Lajmi et al., 2025; Mona et al., 2025; Purcell et al., 2014; Purcell and Brelsford, 2025; Scarparo et al., 2023; Sigeman et al., 2025; Trible et al., 2023; Wang et al., 2013). We performed whole-genome sequencing and comprehensive bioinformatic analyses of the ant *Hypoponera opacior* to test whether wing

development is genetically determined. Our results show that no large genomic element governs winglessness or reproductive morphs in this species. We also found no evidence for a common genetic basis of these morphological differences across the sexes, indicating that the pronounced morphological and behavioural polymorphisms arise through phenotypic plasticity of a shared genome. Only in males was morph associated with several widely distributed SNPs, yet not beyond levels expected by chance, so a genetic component in this sex cannot be excluded.

Supergene regions in ants typically exhibit elevated genetic differentiation (with F_{ST} up to 0.8 – 0.9, reflecting reduced recombination (Brelsford et al., 2020; Gutiérrez-Valencia et al., 2021; Kay et al., 2022; Pracana et al., 2017)). In *H. opacior*, our analyses revealed very low F_{ST} between both morphs, and we did not observe any large regions showing high differentiation in any analysis. These results provide strong evidence against a supergene underlying wing polymorphism in *H. opacior*. Although our GWAS identified several SNPs exceeding the BH-adjusted significance threshold in males, these were scattered across the genome and not more frequent than expected by chance, arguing against a single genetic basis governing wing polymorphism in this species. However, our sampling, including only a handful of genome sequences, cannot exclude more complex sex-specific, polygenic or environmentally modulated genetic contributions. Moreover, a haplotype-resolved assembly from a single individual would provide a more accurate framework for resolving the genetic basis of reproductive polymorphism in *H. opacior*.

The absence of a detectable genetic basis raises an important evolutionary question: how is such morphological diversity maintained? Earlier studies reported that *H. opacior* colonies alternate between outbreeding during mating flights of winged morphs and intranidal mating among ergatoid forms (Foitzik et al., 2011b). Our results show that morph determination does not rely on a single locus or structural variant. We suspect that sexual morphs in *H. opacior* instead reflect an environmentally sensitive developmental switch leading to different, epigenetic profiles, such as histone modifications and DNA methylation (Bonasio et al., 2012). Environmentally induced polyphenism, in which external factors such as larval nutrition, colony size, or seasonal conditions influence developmental pathways (Yang and Andrew Pospisilik, 2019) has

been widely documented in insect species. In addition, some species exhibit developmental regulation via transfer of bioactive molecules to the developing individuals, such as juvenile hormone in *Camponotus floridanus* (LeBoeuf et al., 2016), although larvae in *H. opacior* self-feed and are not known to receive trophallactic provisioning. Other environmental cues relevant to *H. opacior* could include temperature, resource availability, and pheromonal signals within the colony. While our study cannot exclude the possibility that morph determination in *H. opacior* is a polygenic trait such an architecture is unlikely to have evolved. Colonies alternate precisely between reproductive strategies: mating flights of winged sexuals during the monsoon, followed by intranidal mating and colony budding in the fall. This seasonal coordination better fits with the existence of a simple regulatory architecture. Overall, our findings indicate that wing polymorphism in *H. opacior* persists without a supergene, suggesting that plastic or polygenic mechanisms may play a larger role in ant reproductive polymorphisms than currently appreciated. Consistent with this, a preprint by Favreau et al. (2022) found no evidence for supergene control of queen number in *Pheidole pallidula* (Favreau et al., 2022), highlighting that alternative genetic or environmental mechanisms can drive reproductive polymorphisms.

Author contributions

The study was designed by all three authors. Ant collection was carried out by A.L. and S.F. Experimental work and DNA extraction were conducted by A.L., while bioinformatic analyses were performed by A.L. and H.D. A.L. wrote the first draft of the manuscript, which was revised and approved by all authors.

Funding

We would like to thank the German Research Foundation (DFG) –GRK2526/ 1 –Project No. 407023052 and Project DFG FO 298/26-1. Open Access funding enabled and organized by Projekt DEAL.

Acknowledgements

We thank Juliane Hartke for her help in ant collection; Marion Kever and Stefanie Emmling for their support in the laboratory and the Ulrich lab in Mainz for access to their equipment.

Ethics

This study was conducted in accordance with accepted ethical practices for insect research. All efforts were made to minimize disturbance, stress, and harm to the ants during collection and handling. Ant colonies were collected under the collection permit OMB NO. 0596-0082.

Data accessibility statement

Additional supplemental information (figures, tables) can be found in the Supplemental Information. Raw read sequences are accessible through the NCBI BioProject ID PRJNA1309638.

Chapter 5

Supplementary Material

Absence of supergene-linked determination of wing polymorphism and alternative reproductive strategies in the ant *Hypoponera opacior*

Anna Lenhart, Hugo Darras, Susanne Foitzik

Accepted at: Biology Letters (as of February 2026)

Chapter 5

Supplementary Figures

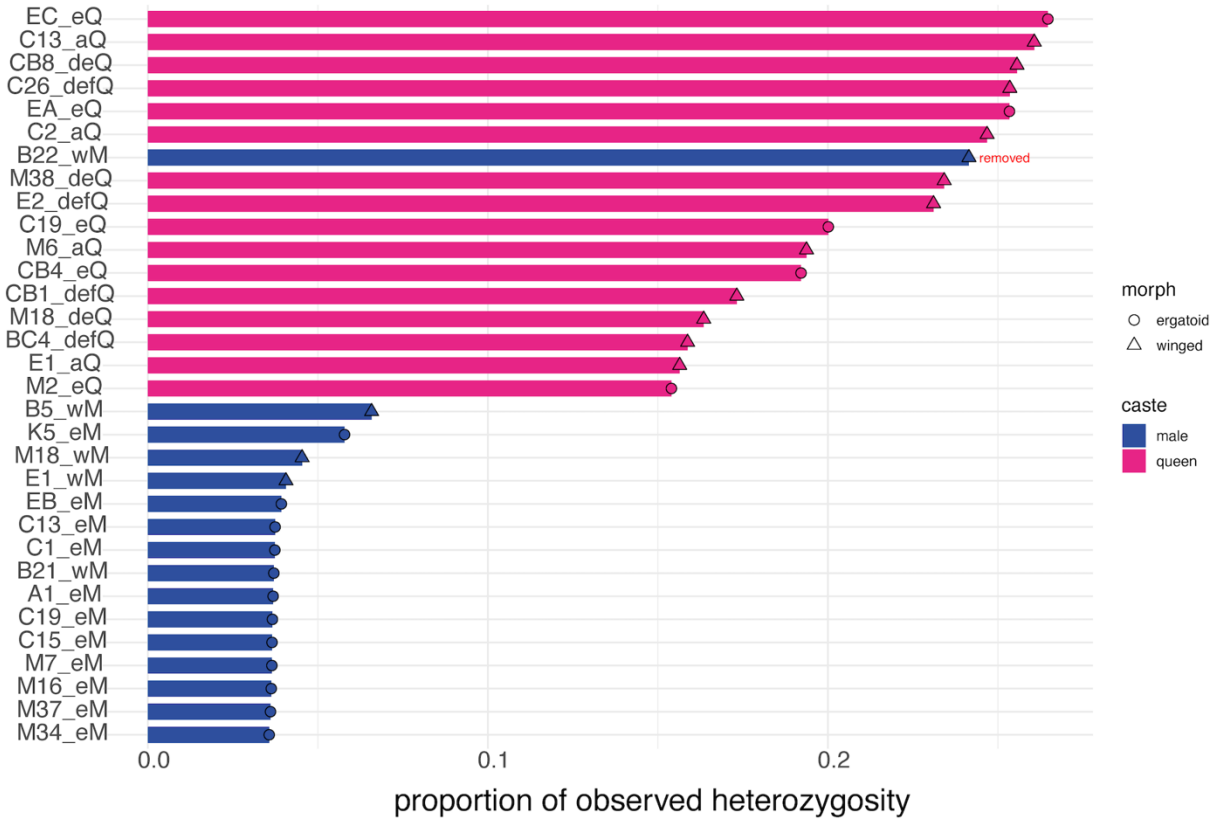

Figure S 1: Proportion of observed heterozygosity (H_o) across individuals, grouped by caste and morph.

Each point represents the proportion of heterozygous sites (1 - (O.HOM. / N_SITES) per individual. Males are shown in blue and queens in pink. The outlier male (B22_wM), identified as diploid based on unusually high heterozygosity, was excluded from downstream analyses.

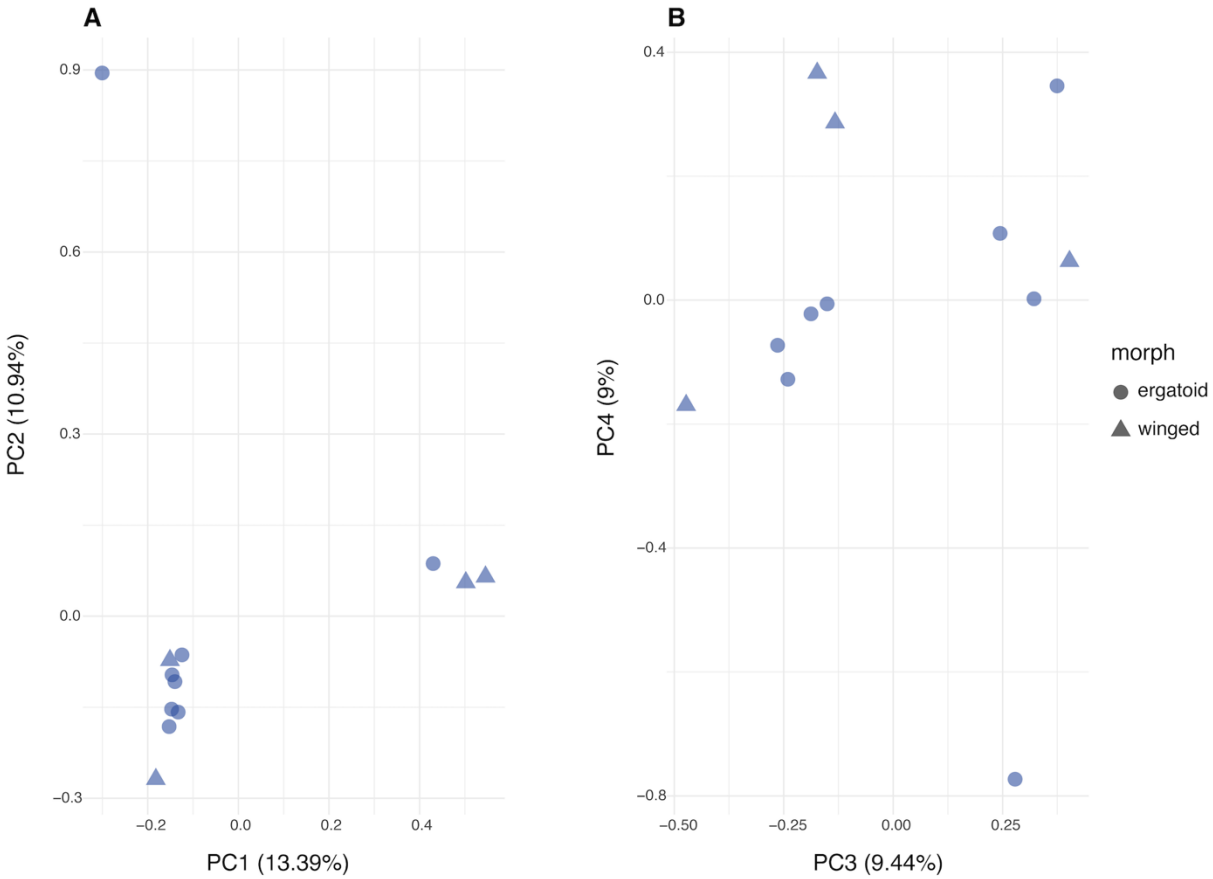

Figure S 2: Principal Component Analysis (PCA) of genetic variation among males classified by morph.

A) Projection of individuals onto PC1 and PC2, which explain 13.39% and 10.94% of the total genetic variance, respectively. **B)** Projection onto PC3 and PC4, explaining 9.44% and 9% of the variance. Each point represents an individual, coloured uniformly, and shaped by morph: circles for ergatoid and triangles for winged males.

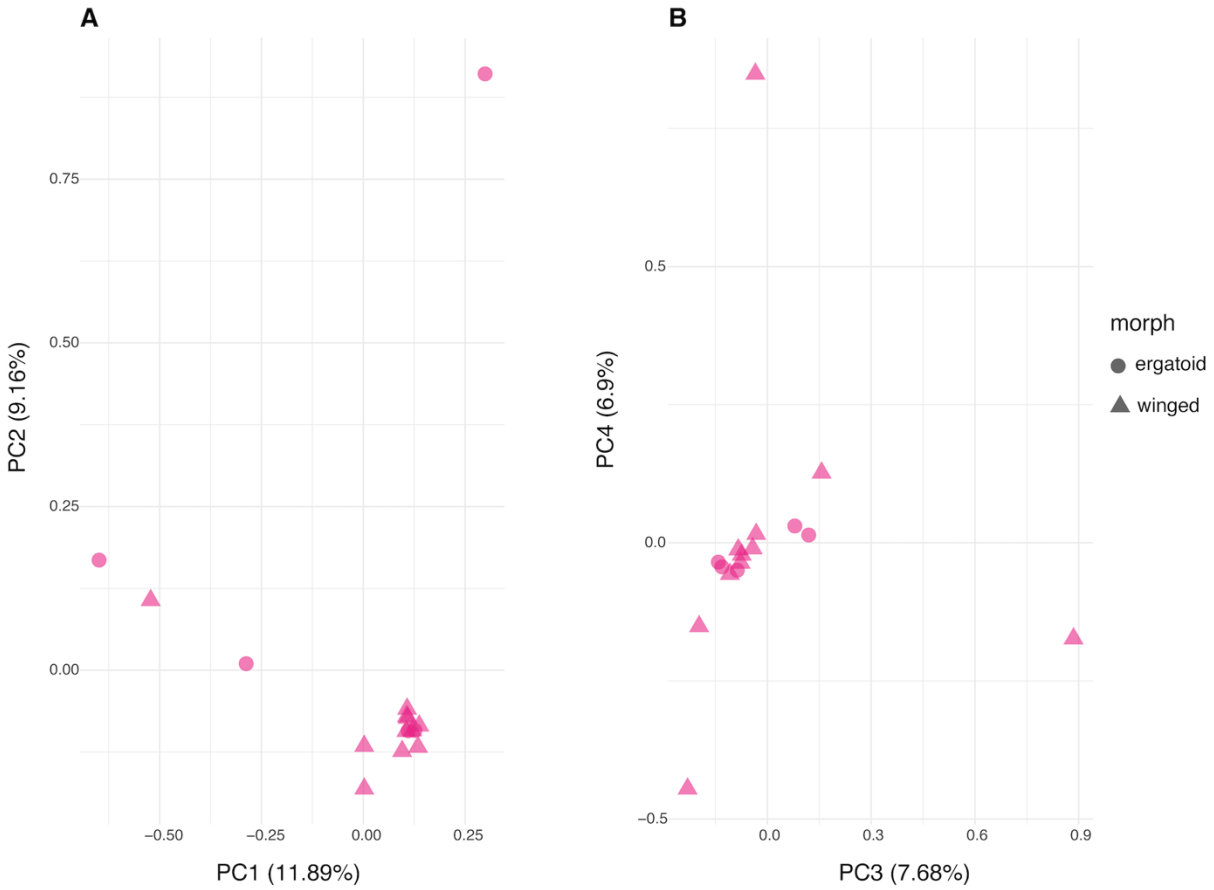

Figure S 3: Principal Component Analysis (PCA) of genetic variation among queens classified by morph.

A) Projection of individuals onto PC1 and PC2, which explain 11.89% and 9.16% of the total genetic variance, respectively. **B)** Projection onto PC3 and PC4, explaining 7.68% and 6.9% of the variance. Each point represents an individual, coloured uniformly, and shaped by morph: circles for ergatoid and triangles for winged queens.

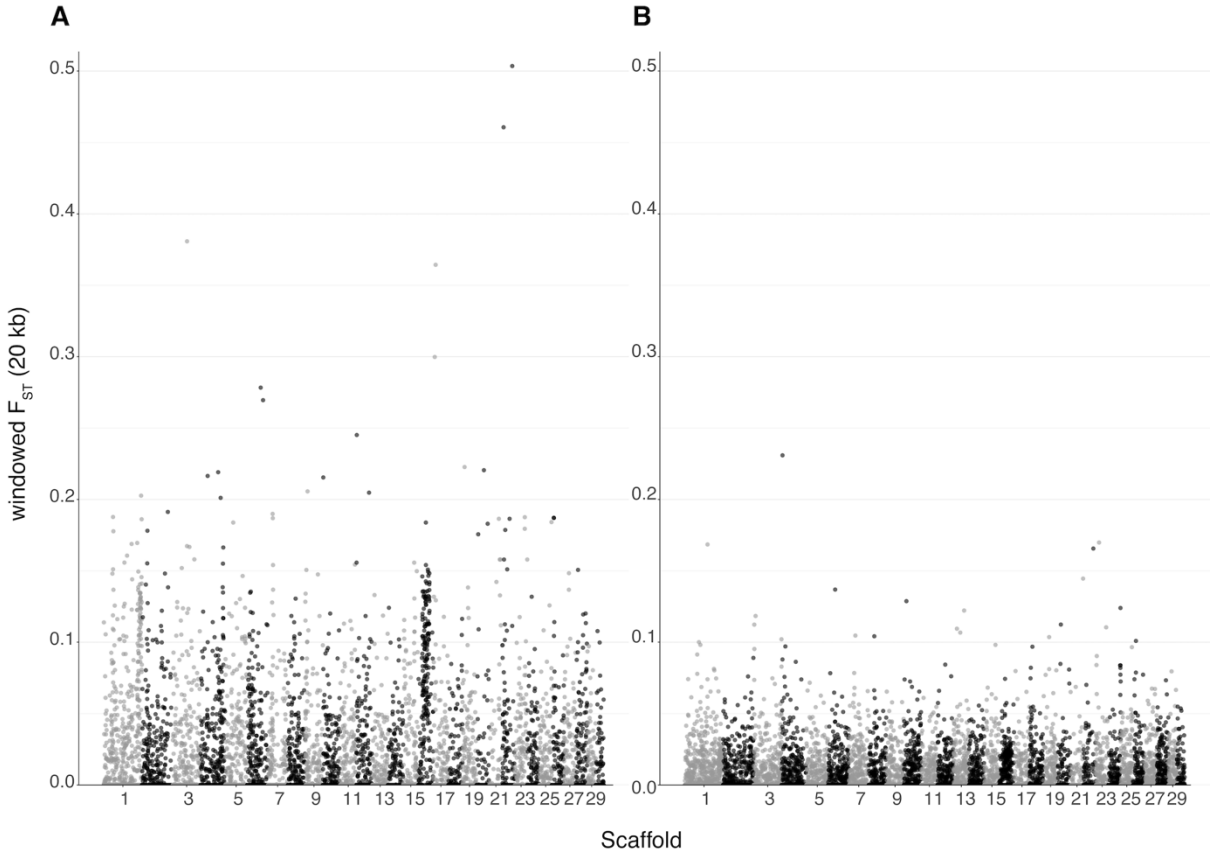

Figure S 4: Windowed F_{ST} along the genome for morph comparisons within sexes.

A) Differentiation between male morphs (ergatoid vs. winged).

B) Differentiation between queen morphs (ergatoid vs. winged).

Each point represents the mean F_{ST} calculated in 20 kb non-overlapping genomic windows, shown across the 30 longest scaffolds. Alternating shading highlights scaffold boundaries. Higher windowed F_{ST} values indicate greater genomic differentiation between the two morphs being compared.

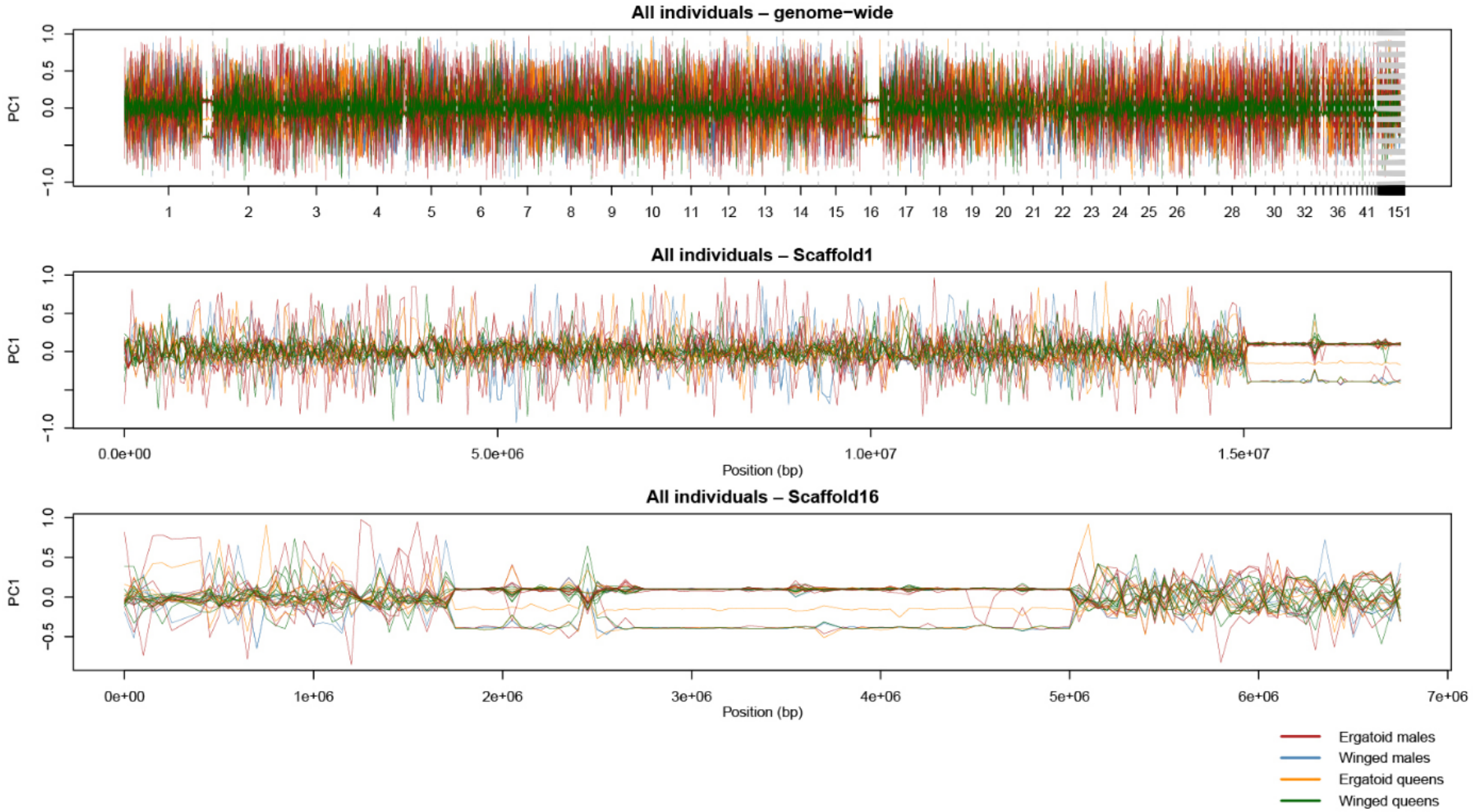

Figure S 5: Rolling PCA in non-overlapping 50 kb windows in ergatoid and winged males and queens.

The first principal component (PC1) is shown on the y-axis. Separate panels show the two scaffolds exhibiting localized linkage disequilibrium.

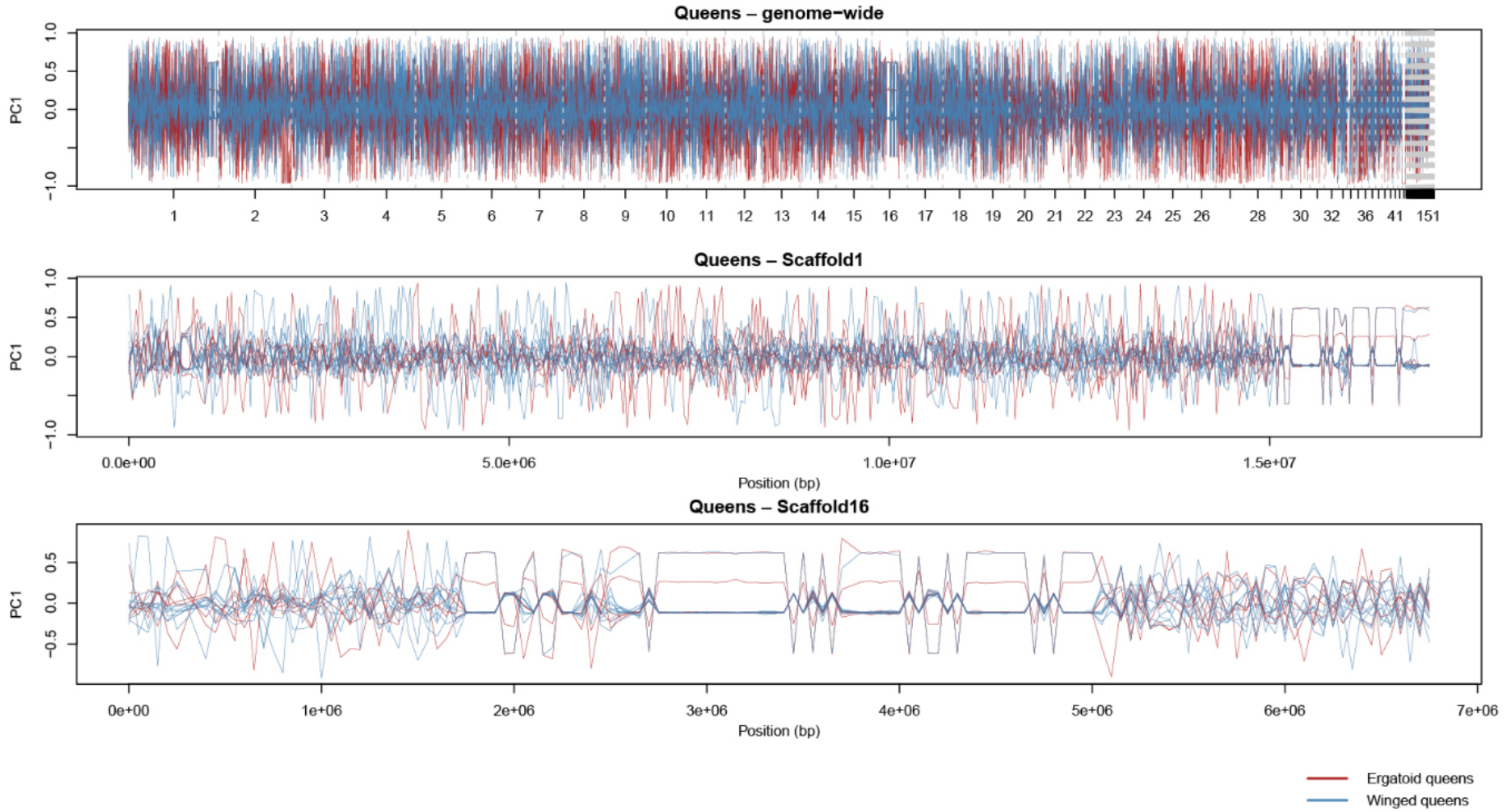

Figure S 6: Rolling PCA in non-overlapping 50 kb windows in ergatoid and winged queens.

The first principal component (PC1) is shown on the y-axis. Separate panels show the two scaffolds exhibiting localized linkage disequilibrium.

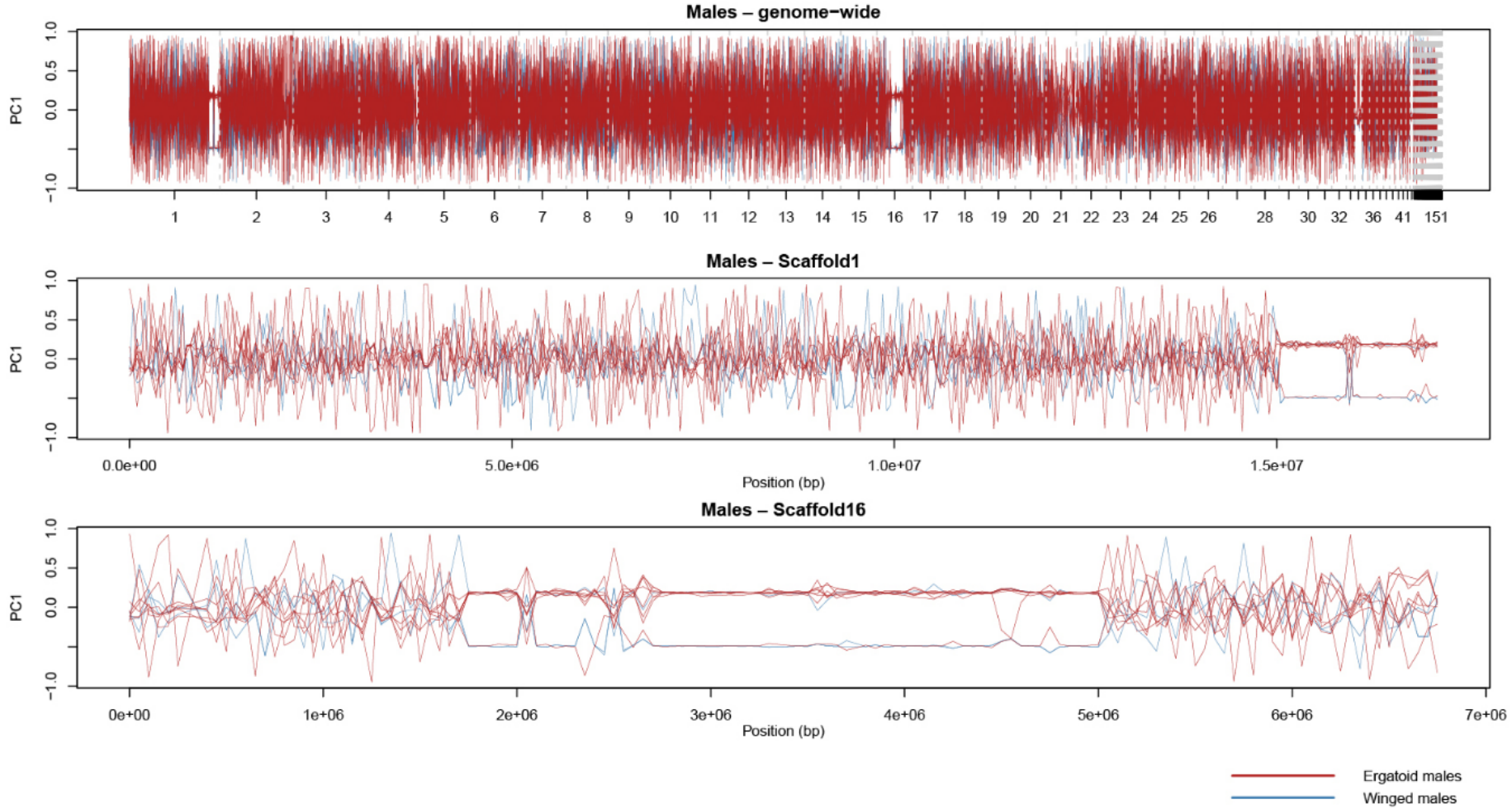

Figure S 7: Rolling PCA in non-overlapping 50 kb windows in ergatoid and winged males.

The first principal component (PC1) is shown on the y-axis. Separate panels show the two scaffolds exhibiting localized linkage disequilibrium.

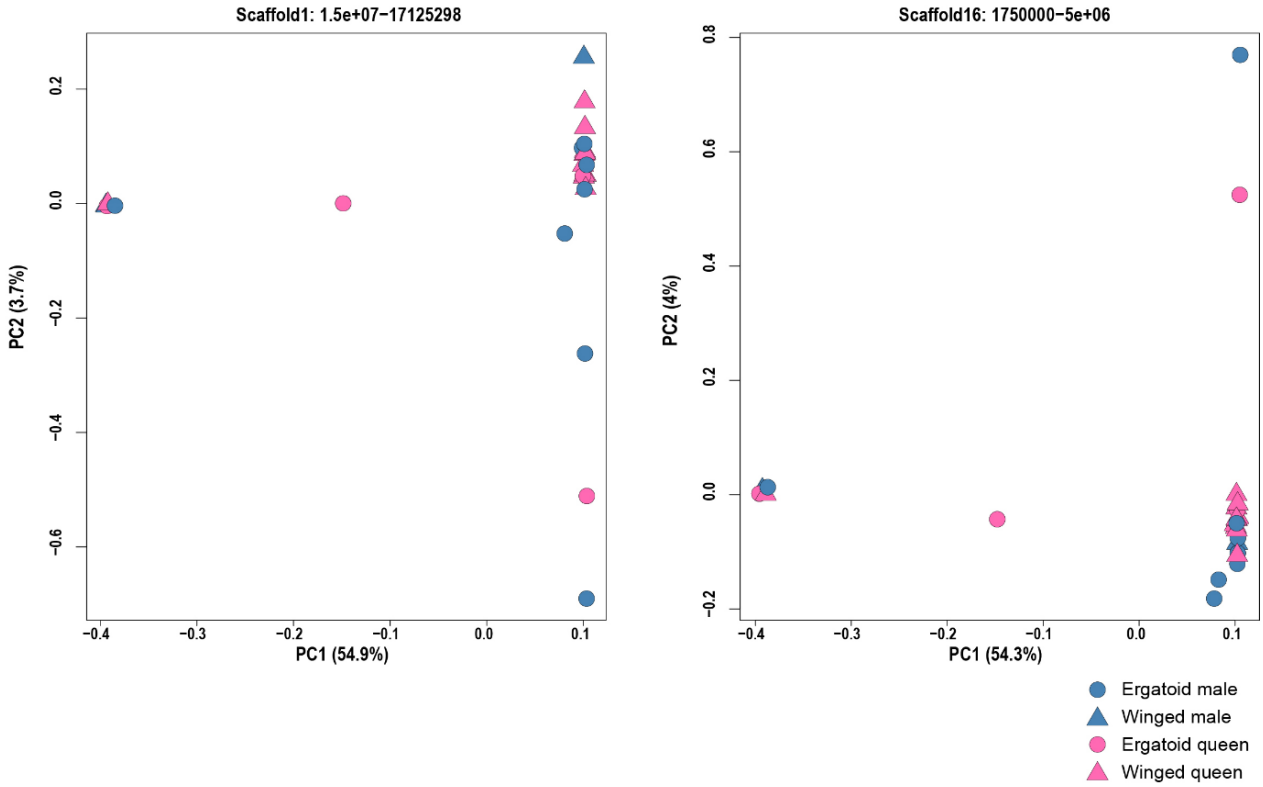

Figure S 8: Principal component analyses of the two genomic regions showing extended linkage in the sliding-window PCA analyses (see Figures S4–S6).

The percentage of variance explained by each principal component is indicated on the axes.

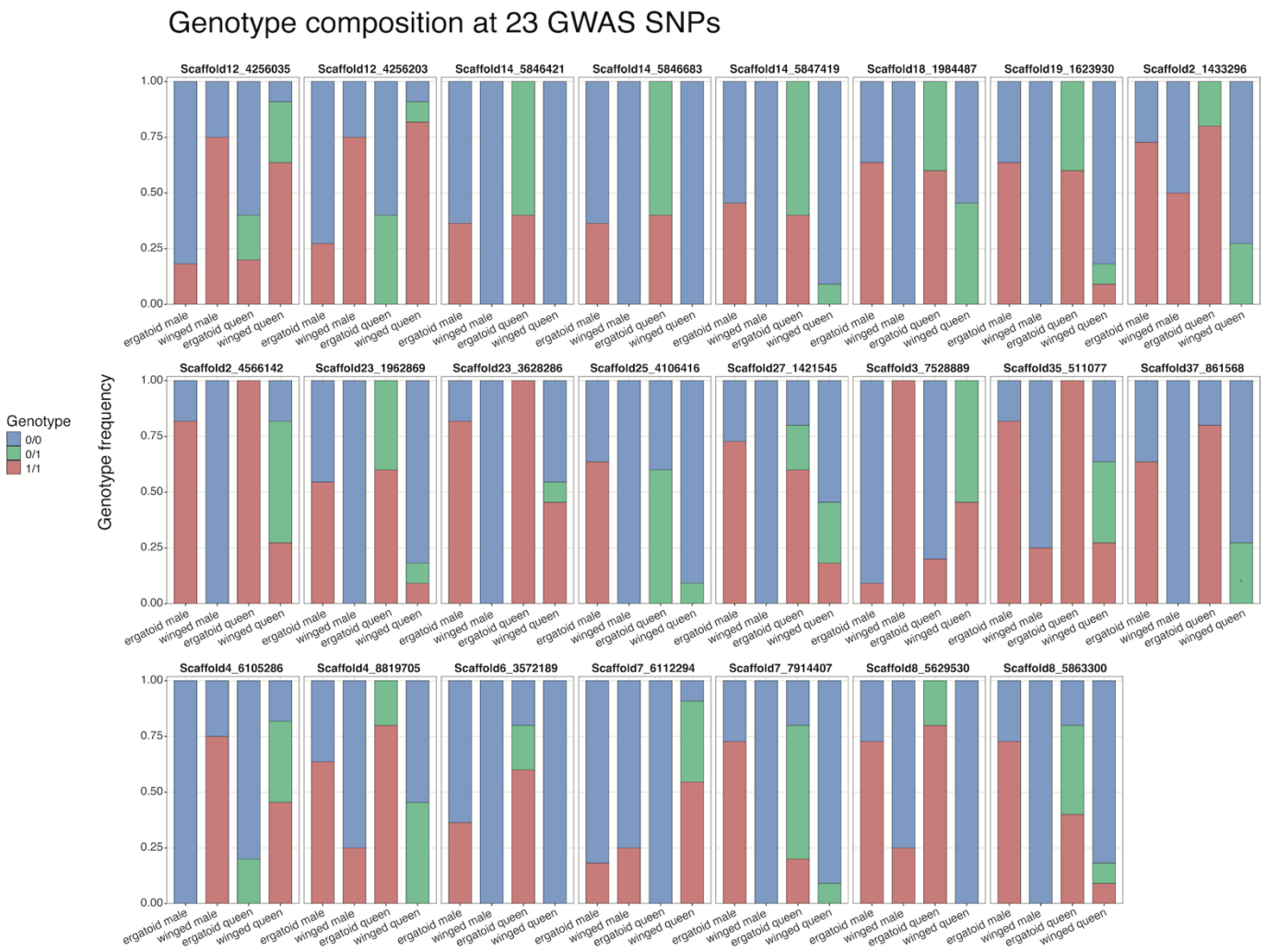

Figure S 9: Genotype patterns of SNPs significantly associated with morph across males and queens. Genotype frequencies for the 23 SNPs that passed a 5% Benjamini–Hochberg false discovery rate (FDR) threshold in the genome-wide association comparison of winged versus ergatoid sexuals. Individuals are grouped by category (ergatoid male, winged male, ergatoid queen, winged queen), and SNPs are organized by scaffold and genomic base position (SNP ID). Color scale denotes genotype frequency (homozygous reference, heterozygous, or homozygous alternate).

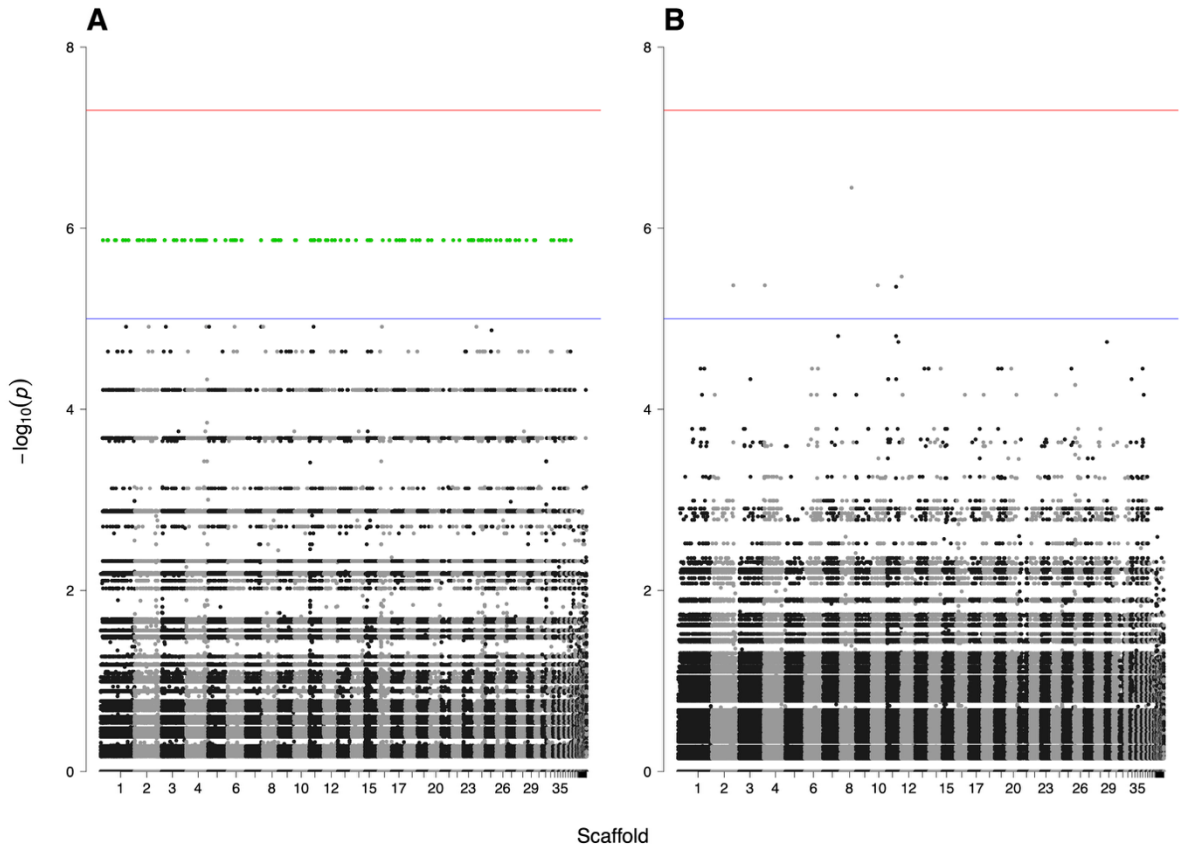

Figure S 10: Manhattan plots of $-\log_{10}$ (p) values from genome-wide association tests for morph differentiation

Manhattan plots showing genome-wide SNP associations with (A) male morphs (ergatoid vs. winged) and (B) queen morphs (ergatoid vs. winged) based on Fisher's exact tests. Each point represents a single SNP, plotted by genomic position across scaffolds. Association significance is shown on the y-axis as –log10 (p-value). Green dots indicate SNPs significant after BH-correction. The solid blue line denotes the conventional suggestive threshold ($p = 10^{-5}$) and the solid red line denotes the conventional genome-wide significance threshold ($p = 5 \times 10^{-8}$). In males, 166 out of 1,054,360 SNPs exceed the BH-significance threshold (BH-adjusted $p \leq 0.05$), whereas no SNPs reach genome-wide significance ($p = 5 \times 10^{-8}$) in either comparison. Alternating black and grey points distinguish adjacent scaffolds.

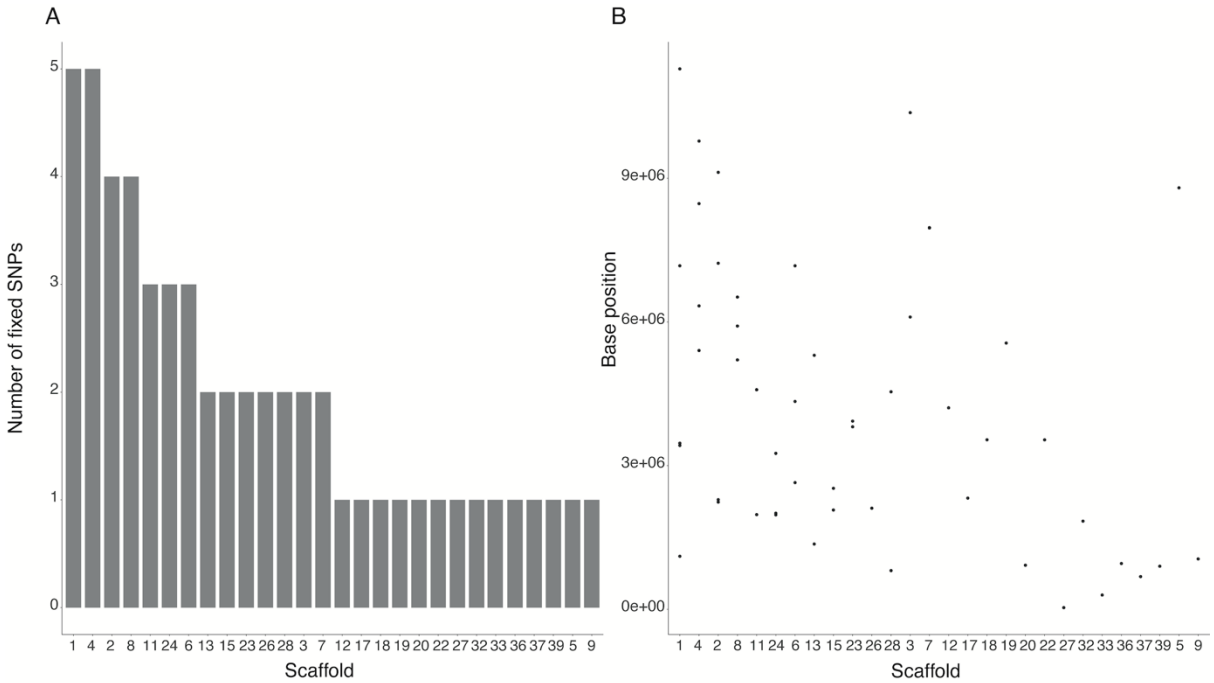

Figure S 11: Genomic distribution of 55 fixed SNPs between male morphs. A) Number of fixed SNPs per scaffold. Scaffolds are ordered by decreasing abundance of fixed SNPs. B) Genomic base positions of individual fixed SNPs mapped to their respective scaffolds, shown in the same scaffold order as in panel A.

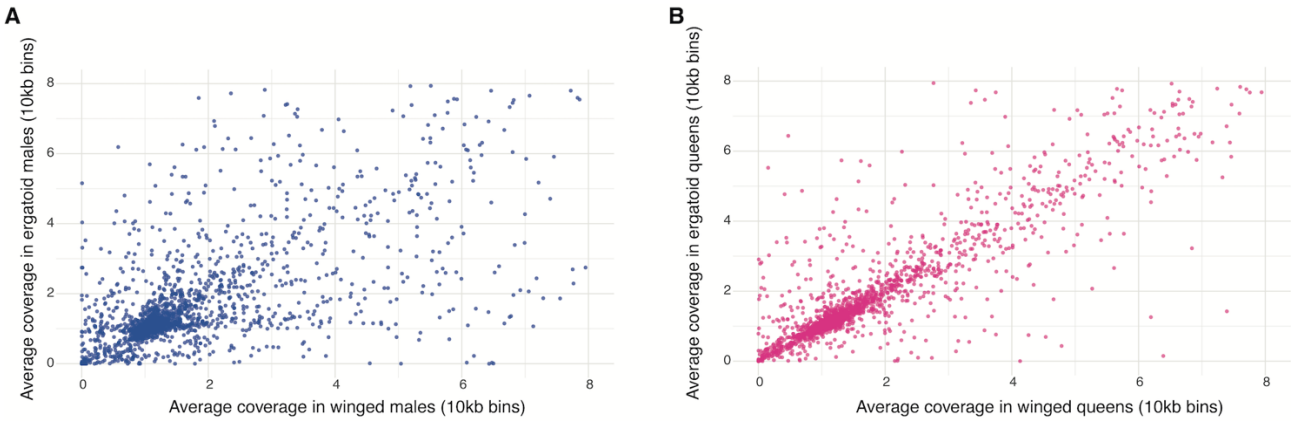

Figure S 12: Normalized genomic coverage comparison between winged and ergatoid morphs in A) males and B) queens.

Each point represents the average normalized read coverage within a 10 kb genomic bin, comparing winged morphs (x-axis) and ergatoid morphs (y-axis). No bins remained significant after Bonferroni correction. The diagonal represents equal coverage between morphs; deviation from the diagonal indicates bin-specific variation in coverage. In males (A), coverage values show more scatter around the diagonal compared to queens (B), likely reflecting increased variability in sequencing depth or biological heterogeneity, though without consistent morph-specific patterns.

Chapter 5

Supplementary Tables

Table S 1: Summary of samples included in this study with colony origin (site, colony ID), collection date and location (at the SWRS field station or in our lab in Mainz, Germany).

No.	Collection site	GPS	Colony ID	Sample ID	Collection date & location	Caste	Morph
1	Chiricahuas, AZ	31.934245, -109.223461	B5	B5_wM	09.07.22, field	male	winged
2	Chiricahuas, AZ	31.934245, -109.223461	B21	B21_wM	09.07.22, field	male	winged
3	Chiricahuas, AZ	31.934245, -109.223461	B22	B22_wM	09.07.22, field	male	winged
4	Chiricahuas, AZ	31.883749, -109.206993	E1	E1_wM	14.07.22, field	male	winged
5	Chiricahuas, AZ	31.985616, -109.407499	M18	M18_wM	10.08.22, lab	male	winged
6	Chiricahuas, AZ	31.882410, -109.176069	K5	K5_eM	18.07.22, field	male	ergatoid
7	Chiricahuas, AZ	31.885336, -109.175248	C1	C1_eM	10.07.22, field	male	ergatoid
8	Chiricahuas, AZ	31.885336, -109.175248	C19	C19_eM	17.07.22, field	male	ergatoid
9	Chiricahuas, AZ	31.885336, -109.175248	C15	C15_eM	17.07.22, field	male	ergatoid
10	Chiricahuas, AZ	31.985616, -109.407499	M16	M16_eM	08.08.22, lab	male	ergatoid
11	Chiricahuas, AZ	31.985616, -109.407499	M37	M37_eM	08.08.22, lab	male	ergatoid
12	Chiricahuas, AZ	31.985616, -109.407499	M34	M34_eM	08.08.22, lab	male	ergatoid
13	Chiricahuas, AZ	31.882523, -109.206350	EB	EB_eM	02.09.22, lab	male	ergatoid
14	Chiricahuas, AZ	31.884125, -109.205946	A1	A1_eM	09.07.22, field	male	ergatoid
15	Chiricahuas, AZ	31.885336, -109.175248	C13	C13_eM	10.07.22, field	male	ergatoid
16	Chiricahuas, AZ	31.985616, -109.407499	M7	M7_eM	19.07.22, field	male	ergatoid
17	Chiricahuas, AZ	31.885336, -109.175248	CB1	CB1_defQ	17.07.22, field	queen	winged
18	Chiricahuas, AZ	31.883749, -109.206993	E2	E2_defQ	14.07.22, field	queen	winged
19	Chiricahuas, AZ	31.885336, -109.175248	C26	C26_defQ	17.07.22, field	queen	winged
20	Chiricahuas, AZ	31.912549, -109.241789	BC4	BC4_defQ	19.07.22, field	queen	winged
21	Chiricahuas, AZ	31.885336, -109.175248	CB8	CB8_deQ	17.07.22, field	queen	winged
22	Chiricahuas, AZ	31.985616, -109.40749	M38	M38_deQ	20.07.22, field	queen	winged

23	Chiricahuas, AZ	31.985616, -109.40749	M18	M18_deQ	10.08.22, lab	queen	winged
24	Chiricahuas, AZ	31.883749, -109.206993	E1	E1_aQ	14.07.22, field	queen	winged
25	Chiricahuas, AZ	31.985616, -109.40749	M6	M6_aQ	19.07.22, field	queen	winged
26	Chiricahuas, AZ	31.885336, -109.175248	C13	C13_aQ	10.07.22, field	queen	winged
27	Chiricahuas, AZ	31.885336, -109.175248	C2	C2_aQ	10.07.22, field	queen	winged
28	Chiricahuas, AZ	31.882523, -109.206350	EC	EC_eQ	14.07.22, field	queen	ergatoid
29	Chiricahuas, AZ	31.985616, -109.40749	M2	M2_eQ	19.07.22, field	queen	ergatoid
30	Chiricahuas, AZ	31.885336, -109.175248	C19	C19_eQ	17.07.22, field	queen	ergatoid
31	Chiricahuas, AZ	31.885336, -109.175248	CB4	CB4_eQ	17.07.22, field	queen	ergatoid
32	Chiricahuas, AZ	31.882523, -109.206350	EA	EA_eQ	14.07.22, field	queen	ergatoid

Table S 2: SNPs significantly associated with morph across males and queens.

List of SNPs passing a 5% Benjamini–Hochberg false discovery rate (FDR) threshold in the genome-wide association test comparing winged and ergatoid morphs. SNP identifiers correspond to genomic positions (scaffold number and BP). P-values are from Fisher's exact tests, and BH-adjusted p-values indicate the FDR-adjusted significance value.

Significant SNP	Scaffold	BP	p-value	BH-adjusted p-value
1	8	5629530	9.82E-09	0.0104
2	3	7528889	3.12E-08	0.0165
3	12	4256203	5.14E-07	0.0361
4	14	5846421	4.75E-07	0.0361
5	14	5846683	4.75E-07	0.0361
6	18	1984487	5.14E-07	0.0361
7	19	1623930	3.53E-07	0.0361
8	2	4566142	4.15E-07	0.0361
9	23	1962869	3.53E-07	0.0361
10	23	3628286	2.41E-07	0.0361
11	35	511077	4.15E-07	0.0361
12	37	861568	3.53E-07	0.0361
13	6	3572189	4.75E-07	0.0361
14	7	6112294	4.15E-07	0.0361
15	8	5863300	3.53E-07	0.0361
16	12	4256035	8.89E-07	0.0408
17	14	5847419	8.56E-07	0.0408
18	2	1433296	8.89E-07	0.0408
19	25	4106416	8.56E-07	0.0408
20	27	1421545	8.89E-07	0.0408
21	4	6105286	8.08E-07	0.0408
22	4	8819705	8.89E-07	0.0408
23	7	7914407	8.56E-07	0.0408
Abbreviations: BP = base position; BH = Benjamini-Hochberg				

Table S 3: Genotype distribution across morphs in *Hypoponera opacior* at 23 significant SNP loci. Genotypes (GT) are shown in VCF format (0/0 = homozygous reference, 0/1 = heterozygous, 1/1 = homozygous alternative). While some genotypes are more frequent in certain morphs, no genotype class segregated exclusively with morph, indicating that these SNPs are unlikely to be causal for morph determination.

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold12_4256035	Scaffold12	4256035	Ergatoid male	0/0	9	11	0.8182
Scaffold12_4256035	Scaffold12	4256035	Ergatoid male	1/1	2	11	0.1818
Scaffold12_4256035	Scaffold12	4256035	Winged male	0/0	1	4	0.25
Scaffold12_4256035	Scaffold12	4256035	Winged male	0/1	0	4	0
Scaffold12_4256035	Scaffold12	4256035	Winged male	1/1	3	4	0.75
Scaffold12_4256035	Scaffold12	4256035	Ergatoid queen	0/0	3	5	0.6
Scaffold12_4256035	Scaffold12	4256035	Ergatoid queen	0/1	1	5	0.2
Scaffold12_4256035	Scaffold12	4256035	Ergatoid queen	1/1	1	5	0.2
Scaffold12_4256035	Scaffold12	4256035	Winged queen	0/0	1	11	0.0909
Scaffold12_4256035	Scaffold12	4256035	Winged queen	0/1	3	11	0.2727
Scaffold12_4256035	Scaffold12	4256035	Winged queen	1/1	7	11	0.6364
Scaffold12_4256203	Scaffold12	4256203	Ergatoid male	0/0	8	11	0.7273
Scaffold12_4256203	Scaffold12	4256203	Ergatoid male	0/1	0	11	0
Scaffold12_4256203	Scaffold12	4256203	Ergatoid male	1/1	3	11	0.2727
Scaffold12_4256203	Scaffold12	4256203	Winged male	0/0	1	4	0.25
Scaffold12_4256203	Scaffold12	4256203	Winged male	0/1	0	4	0
Scaffold12_4256203	Scaffold12	4256203	Winged male	1/1	3	4	0.75
Scaffold12_4256203	Scaffold12	4256203	Ergatoid queen	0/0	3	5	0.6
Scaffold12_4256203	Scaffold12	4256203	Ergatoid queen	0/1	2	5	0.4
Scaffold12_4256203	Scaffold12	4256203	Ergatoid queen	1/1	0	5	0
Scaffold12_4256203	Scaffold12	4256203	Winged queen	0/0	1	11	0.0909
Scaffold12_4256203	Scaffold12	4256203	Winged queen	0/1	1	11	0.0909
Scaffold12_4256203	Scaffold12	4256203	Winged queen	1/1	9	11	0.8182
Scaffold14_5846421	Scaffold14	5846421	Ergatoid male	0/0	7	11	0.6364
Scaffold14_5846421	Scaffold14	5846421	Ergatoid male	0/1	0	11	0
Scaffold14_5846421	Scaffold14	5846421	Ergatoid male	1/1	4	11	0.3636
Scaffold14_5846421	Scaffold14	5846421	Winged male	0/0	4	4	1
Scaffold14_5846421	Scaffold14	5846421	Winged male	0/1	0	4	0

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold14_5846421	Scaffold14	5846421	Winged male	1/1	0	4	0
Scaffold14_5846421	Scaffold14	5846421	Ergatoid queen	0/0	0	5	0
Scaffold14_5846421	Scaffold14	5846421	Ergatoid queen	0/1	3	5	0.6
Scaffold14_5846421	Scaffold14	5846421	Ergatoid queen	1/1	2	5	0.4
Scaffold14_5846421	Scaffold14	5846421	Winged queen	0/0	11	11	1
Scaffold14_5846421	Scaffold14	5846421	Winged queen	0/1	0	11	0
Scaffold14_5846421	Scaffold14	5846421	Winged queen	1/1	0	11	0
Scaffold14_5846683	Scaffold14	5846683	Ergatoid male	0/0	7	11	0.6364
Scaffold14_5846683	Scaffold14	5846683	Ergatoid male	0/1	0	11	0
Scaffold14_5846683	Scaffold14	5846683	Ergatoid male	1/1	4	11	0.3636
Scaffold14_5846683	Scaffold14	5846683	Winged male	0/0	4	4	1
Scaffold14_5846683	Scaffold14	5846683	Winged male	0/1	0	4	0
Scaffold14_5846683	Scaffold14	5846683	Winged male	1/1	0	4	0
Scaffold14_5846683	Scaffold14	5846683	Ergatoid queen	0/0	0	5	0
Scaffold14_5846683	Scaffold14	5846683	Ergatoid queen	0/1	3	5	0.6
Scaffold14_5846683	Scaffold14	5846683	Ergatoid queen	1/1	2	5	0.4
Scaffold14_5846683	Scaffold14	5846683	Winged queen	0/0	11	11	1
Scaffold14_5846683	Scaffold14	5846683	Winged queen	0/1	0	11	0
Scaffold14_5846683	Scaffold14	5846683	Winged queen	1/1	0	11	0
Scaffold14_5847419	Scaffold14	5847419	Ergatoid male	0/0	6	11	0.5455
Scaffold14_5847419	Scaffold14	5847419	Ergatoid male	0/1	0	11	0
Scaffold14_5847419	Scaffold14	5847419	Ergatoid male	1/1	5	11	0.4545
Scaffold14_5847419	Scaffold14	5847419	Winged male	0/0	4	4	1
Scaffold14_5847419	Scaffold14	5847419	Winged male	0/1	0	4	0
Scaffold14_5847419	Scaffold14	5847419	Winged male	1/1	0	4	0
Scaffold14_5847419	Scaffold14	5847419	Ergatoid queen	0/0	0	5	0
Scaffold14_5847419	Scaffold14	5847419	Ergatoid queen	0/1	3	5	0.6
Scaffold14_5847419	Scaffold14	5847419	Ergatoid queen	1/1	2	5	0.4
Scaffold14_5847419	Scaffold14	5847419	Winged queen	0/0	10	11	0.9091
Scaffold14_5847419	Scaffold14	5847419	Winged queen	0/1	1	11	0.0909
Scaffold14_5847419	Scaffold14	5847419	Winged queen	1/1	0	11	0
Scaffold18_1984487	Scaffold18	1984487	Ergatoid male	0/0	4	11	0.3636
Scaffold18_1984487	Scaffold18	1984487	Ergatoid male	0/1	0	11	0

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold18_1984487	Scaffold18	1984487	Ergatoid male	1/1	7	11	0.6364
Scaffold18_1984487	Scaffold18	1984487	Winged male	0/0	4	4	1
Scaffold18_1984487	Scaffold18	1984487	Winged male	0/1	0	4	0
Scaffold18_1984487	Scaffold18	1984487	Winged male	1/1	0	4	0
Scaffold18_1984487	Scaffold18	1984487	Ergatoid queen	0/0	0	5	0
Scaffold18_1984487	Scaffold18	1984487	Ergatoid queen	0/1	2	5	0.4
Scaffold18_1984487	Scaffold18	1984487	Ergatoid queen	1/1	3	5	0.6
Scaffold18_1984487	Scaffold18	1984487	Winged queen	0/0	6	11	0.5455
Scaffold18_1984487	Scaffold18	1984487	Winged queen	0/1	5	11	0.4545
Scaffold18_1984487	Scaffold18	1984487	Winged queen	1/1	0	11	0
Scaffold19_1623930	Scaffold19	1623930	Ergatoid male	0/0	4	11	0.3636
Scaffold19_1623930	Scaffold19	1623930	Ergatoid male	0/1	0	11	0
Scaffold19_1623930	Scaffold19	1623930	Ergatoid male	1/1	7	11	0.6364
Scaffold19_1623930	Scaffold19	1623930	Winged male	0/0	4	4	1
Scaffold19_1623930	Scaffold19	1623930	Winged male	0/1	0	4	0
Scaffold19_1623930	Scaffold19	1623930	Winged male	1/1	0	4	0
Scaffold19_1623930	Scaffold19	1623930	Ergatoid queen	0/0	0	5	0
Scaffold19_1623930	Scaffold19	1623930	Ergatoid queen	0/1	2	5	0.4
Scaffold19_1623930	Scaffold19	1623930	Ergatoid queen	1/1	3	5	0.6
Scaffold19_1623930	Scaffold19	1623930	Winged queen	0/0	9	11	0.8182
Scaffold19_1623930	Scaffold19	1623930	Winged queen	0/1	1	11	0.0909
Scaffold19_1623930	Scaffold19	1623930	Winged queen	1/1	1	11	0.0909
Scaffold23_1962869	Scaffold23	1962869	Ergatoid male	0/0	5	11	0.4545
Scaffold23_1962869	Scaffold23	1962869	Ergatoid male	0/1	0	11	0
Scaffold23_1962869	Scaffold23	1962869	Ergatoid male	1/1	6	11	0.5455
Scaffold23_1962869	Scaffold23	1962869	Winged male	0/0	4	4	1
Scaffold23_1962869	Scaffold23	1962869	Winged male	0/1	0	4	0
Scaffold23_1962869	Scaffold23	1962869	Winged male	1/1	0	4	0
Scaffold23_1962869	Scaffold23	1962869	Ergatoid queen	0/0	0	5	0
Scaffold23_1962869	Scaffold23	1962869	Ergatoid queen	0/1	2	5	0.4
Scaffold23_1962869	Scaffold23	1962869	Ergatoid queen	1/1	3	5	0.6
Scaffold23_1962869	Scaffold23	1962869	Winged queen	0/0	9	11	0.8182
Scaffold23_1962869	Scaffold23	1962869	Winged queen	0/1	1	11	0.0909

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold23_1962869	Scaffold23	1962869	Winged queen	1/1	1	11	0.0909
Scaffold23_3628286	Scaffold23	3628286	Ergatoid male	0/0	2	11	0.1818
Scaffold23_3628286	Scaffold23	3628286	Ergatoid male	0/1	0	11	0
Scaffold23_3628286	Scaffold23	3628286	Ergatoid male	1/1	9	11	0.8182
Scaffold23_3628286	Scaffold23	3628286	Winged male	0/0	4	4	1
Scaffold23_3628286	Scaffold23	3628286	Winged male	0/1	0	4	0
Scaffold23_3628286	Scaffold23	3628286	Winged male	1/1	0	4	0
Scaffold23_3628286	Scaffold23	3628286	Ergatoid queen	0/0	0	5	0
Scaffold23_3628286	Scaffold23	3628286	Ergatoid queen	0/1	0	5	0
Scaffold23_3628286	Scaffold23	3628286	Ergatoid queen	1/1	5	5	1
Scaffold23_3628286	Scaffold23	3628286	Winged queen	0/0	5	11	0.4545
Scaffold23_3628286	Scaffold23	3628286	Winged queen	0/1	1	11	0.0909
Scaffold23_3628286	Scaffold23	3628286	Winged queen	1/1	5	11	0.4545
Scaffold25_4106416	Scaffold25	4106416	Ergatoid male	0/0	4	11	0.3636
Scaffold25_4106416	Scaffold25	4106416	Ergatoid male	0/1	0	11	0
Scaffold25_4106416	Scaffold25	4106416	Ergatoid male	1/1	7	11	0.6364
Scaffold25_4106416	Scaffold25	4106416	Winged male	0/0	4	4	1
Scaffold25_4106416	Scaffold25	4106416	Winged male	0/1	0	4	0
Scaffold25_4106416	Scaffold25	4106416	Winged male	1/1	0	4	0
Scaffold25_4106416	Scaffold25	4106416	Ergatoid queen	0/0	2	5	0.4
Scaffold25_4106416	Scaffold25	4106416	Ergatoid queen	0/1	3	5	0.6
Scaffold25_4106416	Scaffold25	4106416	Ergatoid queen	1/1	0	5	0
Scaffold25_4106416	Scaffold25	4106416	Winged queen	0/0	10	11	0.9091
Scaffold25_4106416	Scaffold25	4106416	Winged queen	0/1	1	11	0.0909
Scaffold25_4106416	Scaffold25	4106416	Winged queen	1/1	0	11	0
Scaffold27_1421545	Scaffold27	1421545	Ergatoid male	0/0	3	11	0.2727
Scaffold27_1421545	Scaffold27	1421545	Ergatoid male	0/1	0	11	0
Scaffold27_1421545	Scaffold27	1421545	Ergatoid male	1/1	8	11	0.7273
Scaffold27_1421545	Scaffold27	1421545	Winged male	0/0	4	4	1
Scaffold27_1421545	Scaffold27	1421545	Winged male	0/1	0	4	0
Scaffold27_1421545	Scaffold27	1421545	Winged male	1/1	0	4	0
Scaffold27_1421545	Scaffold27	1421545	Ergatoid queen	0/0	1	5	0.2
Scaffold27_1421545	Scaffold27	1421545	Ergatoid queen	0/1	1	5	0.2

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold27_1421545	Scaffold27	1421545	Ergatoid queen	1/1	3	5	0.6
Scaffold27_1421545	Scaffold27	1421545	Winged queen	0/0	6	11	0.5455
Scaffold27_1421545	Scaffold27	1421545	Winged queen	0/1	3	11	0.2727
Scaffold27_1421545	Scaffold27	1421545	Winged queen	1/1	2	11	0.1818
Scaffold2_1433296	Scaffold2	1433296	Ergatoid male	0/0	3	11	0.2727
Scaffold2_1433296	Scaffold2	1433296	Ergatoid male	0/1	0	11	0
Scaffold2_1433296	Scaffold2	1433296	Ergatoid male	1/1	8	11	0.7273
Scaffold2_1433296	Scaffold2	1433296	Winged male	0/0	2	4	0.5
Scaffold2_1433296	Scaffold2	1433296	Winged male	0/1	0	4	0
Scaffold2_1433296	Scaffold2	1433296	Winged male	1/1	2	4	0.5
Scaffold2_1433296	Scaffold2	1433296	Ergatoid queen	0/0	0	5	0
Scaffold2_1433296	Scaffold2	1433296	Ergatoid queen	0/1	1	5	0.2
Scaffold2_1433296	Scaffold2	1433296	Ergatoid queen	1/1	4	5	0.8
Scaffold2_1433296	Scaffold2	1433296	Winged queen	0/0	8	11	0.7273
Scaffold2_1433296	Scaffold2	1433296	Winged queen	0/1	3	11	0.2727
Scaffold2_1433296	Scaffold2	1433296	Winged queen	1/1	0	11	0
Scaffold2_4566142	Scaffold2	4566142	Ergatoid male	0/0	2	11	0.1818
Scaffold2_4566142	Scaffold2	4566142	Ergatoid male	0/1	0	11	0
Scaffold2_4566142	Scaffold2	4566142	Ergatoid male	1/1	9	11	0.8182
Scaffold2_4566142	Scaffold2	4566142	Winged male	0/0	4	4	1
Scaffold2_4566142	Scaffold2	4566142	Winged male	0/1	0	4	0
Scaffold2_4566142	Scaffold2	4566142	Winged male	1/1	0	4	0
Scaffold2_4566142	Scaffold2	4566142	Ergatoid queen	0/0	0	5	0
Scaffold2_4566142	Scaffold2	4566142	Ergatoid queen	0/1	0	5	0
Scaffold2_4566142	Scaffold2	4566142	Ergatoid queen	1/1	5	5	1
Scaffold2_4566142	Scaffold2	4566142	Winged queen	0/0	2	11	0.1818
Scaffold2_4566142	Scaffold2	4566142	Winged queen	0/1	6	11	0.5455
Scaffold2_4566142	Scaffold2	4566142	Winged queen	1/1	3	11	0.2727
Scaffold35_511077	Scaffold35	511077	Ergatoid male	0/0	2	11	0.1818
Scaffold35_511077	Scaffold35	511077	Ergatoid male	0/1	0	11	0
Scaffold35_511077	Scaffold35	511077	Ergatoid male	1/1	9	11	0.8182
Scaffold35_511077	Scaffold35	511077	Winged male	0/0	3	4	0.75
Scaffold35_511077	Scaffold35	511077	Winged male	0/1	0	4	0

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold35_511077	Scaffold35	511077	Winged male	1/1	1	4	0.25
Scaffold35_511077	Scaffold35	511077	Ergatoid queen	0/0	0	5	0
Scaffold35_511077	Scaffold35	511077	Ergatoid queen	0/1	0	5	0
Scaffold35_511077	Scaffold35	511077	Ergatoid queen	1/1	5	5	1
Scaffold35_511077	Scaffold35	511077	Winged queen	0/0	4	11	0.3636
Scaffold35_511077	Scaffold35	511077	Winged queen	0/1	4	11	0.3636
Scaffold35_511077	Scaffold35	511077	Winged queen	1/1	3	11	0.2727
Scaffold37_861568	Scaffold37	861568	Ergatoid male	0/0	4	11	0.3636
Scaffold37_861568	Scaffold37	861568	Ergatoid male	0/1	0	11	0
Scaffold37_861568	Scaffold37	861568	Ergatoid male	1/1	7	11	0.6364
Scaffold37_861568	Scaffold37	861568	Winged male	0/0	4	4	1
Scaffold37_861568	Scaffold37	861568	Winged male	0/1	0	4	0
Scaffold37_861568	Scaffold37	861568	Winged male	1/1	0	4	0
Scaffold37_861568	Scaffold37	861568	Ergatoid queen	0/0	1	5	0.2
Scaffold37_861568	Scaffold37	861568	Ergatoid queen	0/1	0	5	0
Scaffold37_861568	Scaffold37	861568	Ergatoid queen	1/1	4	5	0.8
Scaffold37_861568	Scaffold37	861568	Winged queen	0/0	8	11	0.7273
Scaffold37_861568	Scaffold37	861568	Winged queen	0/1	3	11	0.2727
Scaffold37_861568	Scaffold37	861568	Winged queen	1/1	0	11	0
Scaffold3_7528889	Scaffold3	7528889	Ergatoid male	0/0	10	11	0.9091
Scaffold3_7528889	Scaffold3	7528889	Ergatoid male	0/1	0	11	0
Scaffold3_7528889	Scaffold3	7528889	Ergatoid male	1/1	1	11	0.0909
Scaffold3_7528889	Scaffold3	7528889	Winged male	0/0	0	4	0
Scaffold3_7528889	Scaffold3	7528889	Winged male	0/1	0	4	0
Scaffold3_7528889	Scaffold3	7528889	Winged male	1/1	4	4	1
Scaffold3_7528889	Scaffold3	7528889	Ergatoid queen	0/0	4	5	0.8
Scaffold3_7528889	Scaffold3	7528889	Ergatoid queen	0/1	0	5	0
Scaffold3_7528889	Scaffold3	7528889	Ergatoid queen	1/1	1	5	0.2
Scaffold3_7528889	Scaffold3	7528889	Winged queen	0/0	0	11	0
Scaffold3_7528889	Scaffold3	7528889	Winged queen	0/1	6	11	0.5455
Scaffold3_7528889	Scaffold3	7528889	Winged queen	1/1	5	11	0.4545
Scaffold4_6105286	Scaffold4	6105286	Ergatoid male	0/0	11	11	1
Scaffold4_6105286	Scaffold4	6105286	Ergatoid male	0/1	0	11	0

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold4_6105286	Scaffold4	6105286	Ergatoid male	1/1	0	11	0
Scaffold4_6105286	Scaffold4	6105286	Winged male	0/0	1	4	0.25
Scaffold4_6105286	Scaffold4	6105286	Winged male	0/1	0	4	0
Scaffold4_6105286	Scaffold4	6105286	Winged male	1/1	3	4	0.75
Scaffold4_6105286	Scaffold4	6105286	Ergatoid queen	0/0	4	5	0.8
Scaffold4_6105286	Scaffold4	6105286	Ergatoid queen	0/1	1	5	0.2
Scaffold4_6105286	Scaffold4	6105286	Ergatoid queen	1/1	0	5	0
Scaffold4_6105286	Scaffold4	6105286	Winged queen	0/0	2	11	0.1818
Scaffold4_6105286	Scaffold4	6105286	Winged queen	0/1	4	11	0.3636
Scaffold4_6105286	Scaffold4	6105286	Winged queen	1/1	5	11	0.4545
Scaffold4_8819705	Scaffold4	8819705	Ergatoid male	0/0	4	11	0.3636
Scaffold4_8819705	Scaffold4	8819705	Ergatoid male	0/1	0	11	0
Scaffold4_8819705	Scaffold4	8819705	Ergatoid male	1/1	7	11	0.6364
Scaffold4_8819705	Scaffold4	8819705	Winged male	0/0	3	4	0.75
Scaffold4_8819705	Scaffold4	8819705	Winged male	0/1	0	4	0
Scaffold4_8819705	Scaffold4	8819705	Winged male	1/1	1	4	0.25
Scaffold4_8819705	Scaffold4	8819705	Ergatoid queen	0/0	0	5	0
Scaffold4_8819705	Scaffold4	8819705	Ergatoid queen	0/1	1	5	0.2
Scaffold4_8819705	Scaffold4	8819705	Ergatoid queen	1/1	4	5	0.8
Scaffold4_8819705	Scaffold4	8819705	Winged queen	0/0	6	11	0.5455
Scaffold4_8819705	Scaffold4	8819705	Winged queen	0/1	5	11	0.4545
Scaffold4_8819705	Scaffold4	8819705	Winged queen	1/1	0	11	0
Scaffold6_3572189	Scaffold6	3572189	Ergatoid male	0/0	7	11	0.6364
Scaffold6_3572189	Scaffold6	3572189	Ergatoid male	0/1	0	11	0
Scaffold6_3572189	Scaffold6	3572189	Ergatoid male	1/1	4	11	0.3636
Scaffold6_3572189	Scaffold6	3572189	Winged male	0/0	4	4	1
Scaffold6_3572189	Scaffold6	3572189	Winged male	0/1	0	4	0
Scaffold6_3572189	Scaffold6	3572189	Winged male	1/1	0	4	0
Scaffold6_3572189	Scaffold6	3572189	Ergatoid queen	0/0	1	5	0.2
Scaffold6_3572189	Scaffold6	3572189	Ergatoid queen	0/1	1	5	0.2
Scaffold6_3572189	Scaffold6	3572189	Ergatoid queen	1/1	3	5	0.6
Scaffold6_3572189	Scaffold6	3572189	Winged queen	0/0	11	11	1
Scaffold6_3572189	Scaffold6	3572189	Winged queen	0/1	0	11	0

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold6_3572189	Scaffold6	3572189	Winged queen	1/1	0	11	0
Scaffold7_6112294	Scaffold7	6112294	Ergatoid male	0/0	9	11	0.8182
Scaffold7_6112294	Scaffold7	6112294	Ergatoid male	0/1	0	11	0
Scaffold7_6112294	Scaffold7	6112294	Ergatoid male	1/1	2	11	0.1818
Scaffold7_6112294	Scaffold7	6112294	Winged male	0/0	3	4	0.75
Scaffold7_6112294	Scaffold7	6112294	Winged male	0/1	0	4	0
Scaffold7_6112294	Scaffold7	6112294	Winged male	1/1	1	4	0.25
Scaffold7_6112294	Scaffold7	6112294	Ergatoid queen	0/0	5	5	1
Scaffold7_6112294	Scaffold7	6112294	Ergatoid queen	0/1	0	5	0
Scaffold7_6112294	Scaffold7	6112294	Ergatoid queen	1/1	0	5	0
Scaffold7_6112294	Scaffold7	6112294	Winged queen	0/0	1	11	0.0909
Scaffold7_6112294	Scaffold7	6112294	Winged queen	0/1	4	11	0.3636
Scaffold7_6112294	Scaffold7	6112294	Winged queen	1/1	6	11	0.5455
Scaffold7_7914407	Scaffold7	7914407	Ergatoid male	0/0	3	11	0.2727
Scaffold7_7914407	Scaffold7	7914407	Ergatoid male	0/1	0	11	0
Scaffold7_7914407	Scaffold7	7914407	Ergatoid male	1/1	8	11	0.7273
Scaffold7_7914407	Scaffold7	7914407	Winged male	0/0	4	4	1
Scaffold7_7914407	Scaffold7	7914407	Winged male	0/1	0	4	0
Scaffold7_7914407	Scaffold7	7914407	Winged male	1/1	0	4	0
Scaffold7_7914407	Scaffold7	7914407	Ergatoid queen	0/0	1	5	0.2
Scaffold7_7914407	Scaffold7	7914407	Ergatoid queen	0/1	3	5	0.6
Scaffold7_7914407	Scaffold7	7914407	Ergatoid queen	1/1	1	5	0.2
Scaffold7_7914407	Scaffold7	7914407	Winged queen	0/0	10	11	0.9091
Scaffold7_7914407	Scaffold7	7914407	Winged queen	0/1	1	11	0.0909
Scaffold7_7914407	Scaffold7	7914407	Winged queen	1/1	0	11	0
Scaffold8_5629530	Scaffold8	5629530	Ergatoid male	0/0	3	11	0.2727
Scaffold8_5629530	Scaffold8	5629530	Ergatoid male	0/1	0	11	0
Scaffold8_5629530	Scaffold8	5629530	Ergatoid male	1/1	8	11	0.7273
Scaffold8_5629530	Scaffold8	5629530	Winged male	0/0	3	4	0.75
Scaffold8_5629530	Scaffold8	5629530	Winged male	0/1	0	4	0
Scaffold8_5629530	Scaffold8	5629530	Winged male	1/1	1	4	0.25
Scaffold8_5629530	Scaffold8	5629530	Ergatoid queen	0/0	0	5	0
Scaffold8_5629530	Scaffold8	5629530	Ergatoid queen	0/1	1	5	0.2

SNP ID	Scaffold	BP	Category	GT	N[a]	Total[b]	GT frequency
Scaffold8_5629530	Scaffold8	5629530	Ergatoid queen	1/1	4	5	0.8
Scaffold8_5629530	Scaffold8	5629530	Winged queen	0/0	11	11	1
Scaffold8_5629530	Scaffold8	5629530	Winged queen	0/1	0	11	0
Scaffold8_5629530	Scaffold8	5629530	Winged queen	1/1	0	11	0
Scaffold8_5863300	Scaffold8	5863300	Ergatoid male	0/0	3	11	0.2727
Scaffold8_5863300	Scaffold8	5863300	Ergatoid male	0/1	0	11	0
Scaffold8_5863300	Scaffold8	5863300	Ergatoid male	1/1	8	11	0.7273
Scaffold8_5863300	Scaffold8	5863300	Winged male	0/0	4	4	1
Scaffold8_5863300	Scaffold8	5863300	Winged male	0/1	0	4	0
Scaffold8_5863300	Scaffold8	5863300	Winged male	1/1	0	4	0
Scaffold8_5863300	Scaffold8	5863300	Ergatoid queen	0/0	1	5	0.2
Scaffold8_5863300	Scaffold8	5863300	Ergatoid queen	0/1	2	5	0.4
Scaffold8_5863300	Scaffold8	5863300	Ergatoid queen	1/1	2	5	0.4
Scaffold8_5863300	Scaffold8	5863300	Winged queen	0/0	9	11	0.8182
Scaffold8_5863300	Scaffold8	5863300	Winged queen	0/1	1	11	0.0909
Scaffold8_5863300	Scaffold8	5863300	Winged queen	1/1	1	11	0.0909

[a] Number of samples with the respective genotype (VCF format)
[b] The total number of samples for this category
Abbreviations: BP = base position; GT = genotype

General Discussion

Summary of findings

Classical life-history theory predicts a trade-off between reproductive investment and somatic maintenance. Yet, social insects, particularly ants, systematically challenge this expectation: highly fecund queens can live for decades, while genetically identical, non-reproductive workers often survive only weeks or months (Corona et al., 2016; Keller and Genoud, 1997; Keller and Jemielity, 2006). The positive link between reproduction and lifespan is also evident within the worker caste. Reproductively active workers often outlive their non-reproductive nestmates (Blacher et al., 2017; Dixon et al., 2014; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021).

In my dissertation, I addressed this paradox by experimentally manipulating social context and reproductive state and combining physiological and transcriptomic analyses across four ant species spanning a gradient of worker reproductive potential. **Chapter 1** establishes social context and reproductive physiology as key determinants of worker lifespan. *Tapinoma magnum* workers from single-queen colonies lived longer and showed greater ovarian activation than those from two-queen or queenless colonies, and inside workers exhibited higher stress resistance and antioxidant gene expression than outside workers. In contrast, queen age had little effect on fecundity or gene expression, suggesting that queen longevity is buffered against age-related decline. **Chapter 2** provides direct experimental evidence for a reversed longevity/fecundity trade-off mediated by molecular pathways in the clonal raider ant *Ooceraea biroi*. I showed that suppressing reproduction significantly shortened lifespan while transcriptomic analyses revealed that reproductive individuals maintained age-stable expression of genes involved in antioxidant defence, detoxification, and neural maintenance. **Chapter 3** reveals that reproductive activation does not necessarily go hand in hand with sustained somatic maintenance that may confer longevity benefits as queen loss induced ovarian activation and egg laying in *Messor capitatus* without eliciting a survival increase. Instead, survival outcomes were strongly shaped by sub-colony size, with queen loss conferring a survival advantage only in large sub-colonies, while being detrimental in smaller groups. These findings highlight that the translation of reproductive investment into longevity depends on the social and demographic environment. **Chapter 4 and 5**, focused on *Hypoponera opacior*, and revealed that, in an

obligately sterile worker caste, queen removal has no effect on worker survival (**Chapter 4**), suggesting that once reproductive potential is lost, ageing trajectories may become insensitive to social context. In **Chapter 5**, whole-genome resequencing revealed no evidence for a supergene underlying reproductive morph determination, indicating that reproductive and developmental traits in ants can arise through phenotypic plasticity or more dispersed polygenic architectures. Together, these chapters place the ageing results in a broader evolutionary framework, showing how social organisation, reproductive capacity, and genetic architecture jointly shape lifespan variation in social insects.

The impact of reproduction on lifespan and ageing in social insects

Lifespan plasticity does not appear to arise from the activation or suppression of a single conserved pathway. Instead, it reflects the coordinated regulation of multiple processes, including antioxidant defence, cellular repair, fecundity, metabolic buffering, social environment and life history (Li et al., 2023). Consistent with this view, the results of my thesis demonstrate that the relationship between reproduction and longevity is neither fixed nor universal across species. In fact, across four ant species differing in social organisation and worker reproductive potential, lifespan plasticity depends on whether reproductive physiology can be translated into sustained investment in somatic maintenance.

The clearest evidence for a reversal of the classical longevity/fecundity trade-off in social insects emerges from the findings I gathered in Chapter 2. While the TI–J–LiFe framework offers a useful integrative model for interpreting these patterns by linking nutrient-sensing pathways, endocrine signals, and downstream effectors such as vitellogenins and antioxidant genes (Korb et al., 2021), transcriptomic analyses in *O. biroi* revealed that ageing was not associated with consistent changes in canonical components of the juvenile hormone, TOR, or vitellogenin pathways. However, ageing was accompanied by an upregulation of genes associated with serine/threonine kinase activity, suggesting modulation at downstream or interacting regulatory levels. The absence of consistent age-related changes in canonical pathway components does not

contradict a network-based model of lifespan regulation. Rather, it suggests that the observed lifespan effects cannot be explained by a simple up- or downregulation of conserved endocrine pathways at the transcript level. One possible explanation is that previous associations between juvenile hormone, insulin/IGF signalling, or vitellogenin and extended lifespan in social insects may partly reflect confounding factors inherent to comparisons between reproductive and non-reproductive castes, which differ not only in reproductive status but also in morphology, developmental history, and task allocation. By experimentally manipulating reproductive activity within genetically identical workers, my results indicate that the link between fecundity and longevity can be mediated through downstream maintenance processes and pathway interactions rather than uniform shifts in canonical endocrine signalling (Negroni et al., 2021). Similar patterns were observed in *T. magnum* (Chapter 1), where ovarian activation was associated with enhanced oxidative stress resistance accompanied with an upregulation of antioxidant genes, without evidence for broad transcriptional shifts in canonical endocrine pathways. Comparable molecular signatures have been reported in *Temnothorax rugatulus*, where fertile workers upregulate anti-ageing and cellular defence mechanisms (Negroni et al., 2021).

Also, queen age had little effect on fecundity or gene expression profiles, indicating that queens maintain stable physiological performance across age classes. This stability suggests that queen longevity in this species is largely buffered against age-related decline, consistent with the view that queens experience reduced extrinsic mortality and sustained investment in somatic maintenance (Kramer and Schaible, 2013; Lucas and Keller, 2014; Monroy Kuhn et al., 2021; Tasaki et al., 2021). Such stability may persist until shortly before death, when queens exhibit a sudden shift in gene expression, suggesting a compressed or terminal phase of senescence rather than gradual decline (Jaimes-Niño et al., 2022). Thus, *T. magnum* queens appear to occupy a physiologically fixed state optimised for long-term reproduction and survival. In contrast to this queen-specific stability, worker ageing can remain highly plastic when reproductive potential is retained. In totipotent *O. biroi* workers (Chapter 2) experimental suppression of reproduction shifts molecular investment away from protective pathways, accelerating ageing and reducing lifespan. These molecular profiles indicate

that reproductive activity can preserve somatic integrity by sustaining cellular repair, oxidative balance, and neurophysiological function, providing a mechanistic basis for the reversed longevity/fecundity trade-off observed in social insects.

The link between reproductive physiology and longevity is not universal and appears contingent on whether reproductive activation translates into sustained fitness gains. My findings in Chapter 3 (*M. capitatus*) show that queen loss induces ovarian activation and egg laying, yet these reproductive responses do not scale into long-term fitness benefits or consistent lifespan extension. Notably, the effects of queen loss on worker survival were strongly context-dependent and varied with sub-colony size, indicating that reproductive activation in this system is neither uniformly beneficial nor costly for lifespan. These results demonstrate that ovarian activation alone is insufficient to modulate ageing in the absence of conditions that allow reproductive output to translate into lasting fitness returns. The strongest constraints on lifespan plasticity were observed in *H. opacior* (Chapter 4), where workers are obligately sterile and lack ovaries entirely. In this species, queen removal had no effect on worker survival, indicating that ageing trajectories have become largely insensitive to social cues. Viewed from an evolutionary perspective, this pattern is expected when workers have no potential to gain direct fitness through reproduction. Under such conditions, selection for maintaining physiological flexibility after queen loss is weak, and investment in costly maintenance or protective mechanisms is unlikely to be favoured. Instead, selection should optimise workers for efficient task performance over a fixed, short lifespan, rather than for extended survival under rare or unattainable reproductive opportunities. The loss of reproductive potential in this system therefore likely coincides with reduced physiological plasticity, consistent with evidence that strong caste-specific metabolic differentiation and reduced investment in neuroprotective mechanisms restrict the flexibility required for lifespan extension in eusocial insects (Giraldo et al., 2021; Quque et al., 2021).

The species I examined form a spectrum of reproductive and ageing plasticity, ranging from fully reproductively totipotent workers with pronounced lifespan flexibility (*O. biroi*), through species with partial or context-dependent plasticity (*T. magnum*), to

systems in which reproductive activation no longer translates into longevity benefits (*M. capitatus*), and finally to obligately sterile workers with seemingly fixed ageing trajectories (*H. opacior*). This gradient highlights that the uncoupling of reproduction and ageing is not an all-or-nothing feature of sociality, but emerges progressively as reproductive physiology is retained, constrained, or lost. Social evolution has not eliminated the costs of reproduction but has redistributed them through physiological reprogramming and the collective buffering inherent to social life. This interpretation is consistent with recent syntheses linking reproductive longevity to enhanced antioxidant and endocrine coordination (Fernanda Vergara-Martínez et al., 2024; Heinze and Schrempf, 2008; Rodrigues and Flatt, 2016) as well as with patterns reported across diverse social insect species, where reproductive individuals frequently exhibit enhanced somatic maintenance resulting in extended lifespan (Blacher et al., 2017; Dixon et al., 2014; Hartmann and Heinze, 2003; Heinze et al., 2013; Jaimes-Nino et al., 2022; Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021; Negroni et al., 2019).

Social organisation and the evolution of ageing in social insects

Ageing in social insects cannot be understood solely through reproductive physiology. It is also deeply shaped by social organisation, division of labour, and the buffering effects of colony life (Blacher et al., 2017; Dixon et al., 2014; Keller and Genoud, 1997; Keller and Jemielity, 2006; Kramer et al., 2022). In contrast to solitary organisms, where individuals directly bear the costs of environmental stress and extrinsic mortality, social insects experience ageing within a structured social system that redistributes risk, workload, and energetic demands among colony members (Bourke, 2011; Keller and Jemielity, 2006; Koto et al., 2023). Through social buffering, colonies shield certain individuals from environmental stressors, fundamentally altering how and when senescence manifests (Walton et al., 2024). Queens, and in some species reproductive workers, are protected from foraging risks and sustained by the collective labour of nestmates, which reduces extrinsic mortality and allows greater allocation to maintenance and repair (Haight and Liebig, 2025; Heinze and Schrempf, 2008; Keller, 1998; Keller and Genoud, 1997).

General Discussion

One of the key features of social organisation is division of labour, not only between queens and workers, but also within the worker caste. Among workers, division of labour is often expressed as age polyethism, whereby younger workers (i.e., nurses) perform intranidal tasks such as brood care, while older workers (i.e., foragers) transition to extranidal activities including foraging and defence (Blanchard et al., 2000; Chapuisat and Keller, 2002; Hartmann et al., 2019; Wilson, 1980). This behavioural differentiation is tightly linked to ageing trajectories. In many social insect species, foragers experience higher extrinsic mortality, elevated metabolic demands, and increased exposure to oxidative stress, pathogens, and predators compared to nurses (Bernadou et al., 2020; Beros et al., 2021; Münch and Amdam, 2010; Omholt and Amdam, 2004). As a consequence, task allocation alone can generate substantial lifespan variation within a caste, independent of chronological age. The findings from Chapter 1 illustrate how task allocation interacts with physiology to shape ageing (Lenhart et al., 2025), consistent with patterns reported in other ant species (Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021). These task-related differences were not merely behavioural but were coupled to reproductive physiology: workers with greater ovarian activation showed improved physiological condition and survival. Intranidal individuals benefit from a buffered nest environment, stable microclimatic conditions, and reduced energetic expenditure, all of which likely contribute to enhanced stress resistance and longevity (Giraldo and Traniello, 2014; Heinze and Giehr, 2021; Helft et al., 2012; Moroń et al., 2008; Quque et al., 2023, 2021; Rueppell et al., 2007). Such buffering effects help explain why ageing trajectories in social insects often differ dramatically from those observed in solitary taxa exposed to unmitigated environmental challenges. This suggests that social environment, task, reproductive state, and somatic maintenance form an integrated physiological framework rather than independent axes of variation.

However, comparisons among the species studied here indicate that social buffering alone is insufficient to explain the diversity of ageing responses, and that behavioural protection must coincide with physiological capacity to translate social context into molecular pathways supporting somatic maintenance. In *O. biroi* (Chapter 2), reproductive workers maintained molecular pathways associated with antioxidant defence, detoxification, and neural maintenance with age. By contrast, in *M. capitatus*

(Chapter 3), queen loss induced ovarian activation and changes in social organisation, yet these shifts did not translate into extended lifespan. This suggests that while social context can trigger reproductive responses, it does not necessarily engage the physiological mechanisms required to buffer ageing. The limits of social buffering are most evident in *H. opacior* (Chapter 4), where ageing trajectories remained unchanged despite potential changes in social dynamics following queen loss.

From an evolutionary perspective, these patterns reflect the dual action of selection at individual and colony levels, where fitness is often realised indirectly through kin rather than personal reproduction (Hamilton, 1964). Within the superorganism framework, queens function as the reproductive germline of the colony, while workers collectively constitute the soma (Boomsma and Gawne, 2018; Wheeler, 1911). This partitioning of reproductive and somatic functions allows queens to achieve both extreme fecundity and exceptional longevity, as the energetic costs of reproduction are buffered by workers and exposure to extrinsic mortality is minimised (Keller and Genoud, 1997; Keller and Jemielity, 2006). Moreover, this organisation is expected to generate distinct selective pressures on somatic maintenance across castes. Ageing within organisms is often tissue-specific (López-Otín et al., 2013; Maegawa et al., 2010), with selective protection of functionally critical tissues. Analogously, selection at the colony level may favour sustained investment in maintenance and longevity in queens, while permitting faster ageing in workers whose fitness contribution is primarily task-based and time-limited. In this sense, caste-specific ageing patterns can be viewed as a modular allocation of maintenance within the colony, rather than as uniform organism-wide ageing. The results of my thesis refine this framework by showing that, under specific conditions, analogous but attenuated principles can apply to workers, depending on their reproductive potential and social role. In *O. biroi*, selection likely favours physiological architectures that couple reproductive activity to somatic maintenance, as worker survival directly contributes to colony fitness. In contrast, in species where worker reproduction is facultative or infrequent and does not reliably translate into sustained fitness gains (*e.g., M. capitatus*), or is entirely absent (*H. opacior*), this link appears weakened or lost.

These patterns align with comparative evidence indicating that the degree of fertility predicts lifespan plasticity across ants and other eusocial insects (Elsner et al., 2018; Feldmeyer et al., 2014; Heinze et al., 2013; Heinze and Giehr, 2021; Korb, 2016; Lin et al., 2021; Negroni et al., 2021b). Rather than representing a universal feature of eusociality, the reversal of the longevity/fecundity trade-off appears contingent on the evolutionary stability and functional relevance of worker reproduction within a species' life history. Where worker reproduction contributes meaningfully to colony fitness, selection may favour mechanisms that mitigate its somatic costs. Conversely, when reproductive activation yields limited or inconsistent fitness returns, selection for such mechanisms is expected to be weak or absent.

Genetic architectures of reproduction and their implications for ageing

While Chapters 1–4 demonstrate that ageing and lifespan in social insects are shaped by reproductive physiology, social context, and life history, Chapter 5 addresses a complementary question: to what extent are reproductive strategies genetically determined? In several ant species, alternative social organisations and reproductive strategies are controlled by supergenes that lock together suites of traits related to reproductive behaviour, queen number, and queen size (Braim, 2015; Brelsford et al., 2020; De Gasperin et al., 2025; Errbii et al., 2022; Kay et al., 2022; Lagunas-Robles et al., 2021; Lajmi et al., 2025; Pracana et al., 2017; Scarparo et al., 2023; Sigeman et al., 2025; Trible et al., 2023; Wang et al., 2013). By genetically fixing for example social structure (*i.e.,* monogyny vs. polygyny), supergenes can stabilise coordinated suites of behavioural, physiological, and life-history traits. Across ant species, monogynous and polygynous colonies often differ not only in social structure but also in behaviour, ageing patterns, stress resistance, and lifespan (Heinze, 2017; Negroni et al., 2021; Ross and Keller, 1995; Wang et al., 2013). Consistent with this association, monogynous queens in many ants achieve exceptional longevity, sometimes living for decades, whereas queens in polygynous systems typically exhibit shorter lifespans and reduced investment in long-term somatic maintenance (Goodisman and Ross, 1999; Hölldobler

and Wilson, 1990; Janet, 1907; Keller, 1998, 1995; Lenhart et al., 2025; Oettler and Schrempf, 2016; Reuter et al., 2001; Schrempf et al., 2011). Thus, if social and reproductive traits become genetically stabilised – whether through supergenes or other forms of genetic canalisation – ageing trajectories may likewise become constrained, reducing the scope for plastic responses to environmental or social variation.

Against this background, the results from *H. opacior* (Chapter 5) are particularly informative. Despite exhibiting striking alternative reproductive morphs in both sexes that correlate with monogynous and polygynous colony organisation (Foitzik et al., 2011a, 2010, 2002; Kureck et al., 2012), genomic analyses revealed no evidence for a supergene underlying sexual morph determination. Instead, the development of male and queen morphs appears to be governed by environmentally sensitive developmental plasticity and/or a more dispersed, recombining polygenic basis, rather than by a single large non-recombining genomic region such as a supergene. This absence of genetic canalisation of reproductive morphs contrasts with supergene-based research in social insects, where large non-recombining regions are most often associated with social organisation, queen number, or recognition systems, but in some cases also underlie morphological polymorphisms. For example, in several ant species, including *Harpagoxenus sublaevis, Leptothorax sp. A*, and *Myrmecina graminicola*, queen wing polymorphisms are determined by a single locus or a supergene (Buschinger, 1978; Buschinger and Schreiber, 2002; Heine and Buschinger, 1989; Mona et al., 2025). Similar genetic control of wing polymorphisms has also been described in other insects, such as stoneflies and male aphids (Li et al., 2020; Veale et al., 2018). In contrast, the lack of any such genomic coupling in *H. opacior*, despite pronounced reproductive morphs, suggests that reproductive diversity in this species remains developmentally flexible rather than genetically fixed. This finding provides an important counterpoint to the ageing patterns observed in Chapter 4: while worker lifespan and ageing trajectories in *H. opacior* appear insensitive to social context, the determination of reproductive morphs remains developmentally flexible rather than genetically canalised by a supergene. This contrast highlights that ageing regulation, caste or morph determination, and social structure represent distinct evolutionary dimensions that do not need to share a common genetic basis.

Interpretive scope and alternative explanations

By integrating experimental, physiological, and transcriptomic approaches, my thesis offers a comprehensive view of ageing in social insects; however, several limitations and alternative interpretations should be acknowledged. First, the molecular inferences regarding the relationship between reproductive activity and somatic maintenance are based on transcriptomic data from *T. magnum* (Chapter 1) and *O. biroi* (Chapter 2). In contrast, conclusions drawn from *M. capitatus* (Chapter 3) and *H. opacior* (Chapter 4) rely primarily on survival data and ovarian assessments. As a result, mechanistic interpretations for these latter species are necessarily indirect and should be viewed as hypotheses to be tested by future molecular analyses. Second, all experiments were conducted under controlled laboratory conditions, which simplify the ecological complexity experienced by ants in natural environments. Factors such as fluctuating resource availability, pathogen exposure, predation risk, and colony demography may substantially influence both reproductive dynamics and ageing trajectories. Consequently, while laboratory experiments are essential for establishing causality, the magnitude and direction of lifespan effects observed here may differ in the field. Third, experimental manipulations of social context, particularly queen removal and brood manipulation, may induce physiological stress that itself affects survival. Although similar manipulations were applied across treatments and species, the intensity and consequences of such stress may vary depending on species-specific life histories and social organisation. However, the contrasting outcomes observed across species, most notably the shortened lifespan following reproductive suppression in *O. biroi* versus the absence of lifespan effects in *M. capitatus* and *H. opacior*, suggest that stress alone cannot account for the observed patterns. Fourth, ageing is a multifaceted process that unfolds across tissues and timescales. The tissue-specific nature of ageing further suggests that lifespan modulation in social insects may be achieved through selective protection of key tissues, such as the brain or fat body, rather than through uniform organism-wide changes, a pattern consistent with delayed senescence rather than its absence. However, other tissues, such as the gut or reproductive tissues, may also contribute to lifespan variation (Anderson et al., 2018; Chen et al., 2024). Similarly, post-transcriptional regulation, protein turnover, and metabolic fluxes were not assessed and

may decouple gene expression from functional outcomes. Finally, my thesis is limited to four ant species. Phylogenetic history, ecological niche, and colony life-history strategy may all influence ageing independently of reproductive physiology. Expanding this framework to additional species would help determine the generality of the patterns I identified here. Despite these limitations, the convergence of results across independent experimental systems strengthens my central conclusions. Even in the absence of transcriptomic data for all species, the consistent association between reproductive potential, social context, and lifespan plasticity supports the view that ageing in social insects is shaped by interactions between physiology, social organisation, and evolutionary constraints.

Conclusion

My dissertation reveals a spectrum of ageing strategies shaped by reproductive potential and social organisation. Rather than representing a single, universal outcome of sociality, ageing plasticity emerges as a lineage-specific property that reflects how reproductive roles are distributed, stabilised, or lost within colonies. Across this spectrum, reproductive activity influences lifespan only when reproductive physiology is linked to sustained investment in somatic maintenance. In reproductively totipotent systems such as *Ooceraea biroi*, continuous or cyclic reproduction maintain longevity, while experimental suppression of reproduction accelerated ageing, demonstrating that reproductive inactivity, rather than reproductive effort itself, can drive somatic decline. In contrast, species in which worker reproduction is present but not consistently coupled to long-term somatic maintenance show progressively reduced lifespan plasticity. In *Tapinoma magnum*, workers exhibited context-dependent physiological plasticity shaped by task allocation, whereas queens maintained stable physiological performance across age classes, consistent with strong buffering against age-related decline. In *Messor capitatus*, ovarian activation occurred but translated into survival effects only in a context-dependent manner, varying with sub-colony size and failing to produce a consistent longevity benefit across conditions, whereas in *Hypoponera opacior,* obligate worker sterility coincided with ageing trajectories that were entirely insensitive to social or reproductive cues.

I show that lifespan plasticity does not arise from uniform changes in conserved endocrine pathways, but from context-dependent regulation of downstream maintenance processes, including antioxidant defence, cellular repair, and neural integrity. These findings support the view that social evolution has not eliminated the costs of reproduction but has redistributed and mitigated them through physiological reprogramming and collective buffering at the colony level. Where reproductive physiology remains functionally integrated with somatic maintenance, ageing remains plastic; where this integration is partial, inconsistent, or evolutionarily lost, ageing becomes constrained. I identify physiological capacity as a key limiting factor in the social modulation of ageing. Social organisation can reduce extrinsic mortality and structure exposure to risk through division of labour and social buffering, but only

individuals that retain the physiological machinery to translate reproductive activation into sustained somatic maintenance can realise longevity benefits. Once this capacity is lost, social context alone is insufficient to modify ageing trajectories, even in socially flexible systems.

The findings I gathered for my dissertation advance our understanding of ageing as an evolvable, socially embedded life-history trait. Social insects do not escape ageing, but they reveal how its timing, expression, and fitness consequences can be reshaped by the interaction between physiology, social organisation, and evolutionary history. By explicitly linking reproductive potential, physiological integration, and lifespan plasticity, my thesis provides a framework for investigating how social systems modify fundamental ageing processes, with implications that extend beyond social insects to broader questions of life-history evolution and the biology of ageing.

Future research perspectives

The findings of my thesis open several avenues for future research aimed at disentangling how reproductive potential, social organisation, and genetic architecture interact to shape ageing in social insects. An immediate extension would be to complement the physiological data from *M. capitatus* (Chapter 3) and *H. opacior* (Chapter 4) with molecular analyses. Transcriptomic or proteomic profiling of workers following queen removal in these species would allow direct testing of whether the absence of lifespan differences after queen removal reflects a failure to activate/inactivate somatic maintenance pathways or instead arises from downstream constraints such as post-transcriptional regulation or tissue-specific ageing processes. Such data would be essential to determine whether the molecular signatures observed in reproductively active and inactive *O. biroi* workers represent a generalizable mechanism or a lineage-specific phenomenon. Future work should expand the comparative framework to include additional species spanning varying stages of worker reproductive potential and social complexity. Integrating phylogenetic comparative methods could further disentangle the relative contributions of ancestry, ecology, and social structure to lifespan evolution. Extending analyses beyond transcriptomics will be

crucial for understanding the mechanistic basis of ageing plasticity. While gene expression analyses provide valuable insights into regulatory changes, functional outcomes ultimately depend on protein abundance, metabolic flux, and cellular repair processes (Huang et al., 2025; Musselman et al., 2025; Quque et al., 2023). Combining transcriptomic data with metabolomics, measures of oxidative damage, immune function, or mitochondrial performance would provide a more comprehensive picture of how somatic maintenance is sustained, or fails to be sustained, across reproductive states and social contexts. Moreover, experimental manipulation of specific candidate pathways represents a promising direction for future research. Targeted perturbations of antioxidant systems, nutrient-sensing pathways, or reproductive signalling using pharmacological treatments or RNA interference where feasible could establish causal links between molecular regulation and lifespan outcomes (Ihle et al., 2019; Nilsen et al., 2011). Such approaches would be particularly informative in species like *O. biroi*, where reproduction can be experimentally decoupled from age, morphology and genetic background. Finally, the genomic results from *H. opacior* (Chapter 5) highlight the need for broader investigation into how genetic architecture constrains life-history plasticity. Future studies comparing species with supergene-determined social organisation to those with developmentally plastic reproductive strategies could reveal whether genetic canalisation limits ageing flexibility. Studies integrating genomics and lifespan data would be especially valuable for understanding how genetic and physiological constraints co-evolve.

Acknowledgments

Acknowledgments

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References

References

Ahn SJ, Marygold SJ. 2021. The UDP-Glycosyltransferase family in Drosophila melanogaster: Nomenclature update, gene expression and phylogenetic analysis. Frontiers in Physiology Volume 12-2021. DOI: 10.3389/fphys.2021.648481

Alexa A, Rahnenführer J. 2024. topGO: Enrichment analysis for gene ontology. DOI: https://doi.org/10.18129/B9.bioc.topGO

Alexander RD. 1974. The evolution of social behavior. *Annual Review of Ecology and Systematics* **5**:325–383. See: https://courses.washington.edu/ccab/Alexander1974.pdf

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of molecular biology* **215**:403–410. DOI: 10.1016/S0022-2836(05)80360-2

Amsalem E, Malka O, Grozinger C, Hefetz A. 2014. Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. *BMC Evolutionary Biology* **14**:45. DOI: https://doi.org/10.1186/1471-2148-14-45

Anderson KE, Ricigliano VA, Mott BM, Copeland DC, Floyd AS, Maes P. 2018. The queen's gut refines with age: longevity phenotypes in a social insect model. *Microbiome* **6**:108. DOI: https://doi.org/10.1186/s40168-018-0489-1

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. See: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Aron S, Keller L, Passera L. 2001. Role of resource availability on sex, caste and reproductive allocation ratios in the Argentine ant *Linepithema humile*. J*ournal of Animal Ecology* **70**:831–839. DOI: https://doi.org/10.1046/j.0021-8790.2001.00545.x

Arrese EL, Soulages JL. 2010. Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology*. DOI: https://doi.org/10.1146/annurev-ento-112408-085356

Austad SN. 2009. Comparative biology of aging. T*he Journals of Gerontology: Series A* **64A**:199–201. DOI: https://doi.org/10.1093/gerona/gln060

Avril A, Purcell J, Brelsford A, Chapuisat M. 2019. Asymmetric assortative mating and queen polyandry are linked to a supergene controlling ant social organization. *Molecular Ecology* **28**:1428–1438. DOI: https://doi.org/10.1111/mec.14793

References

Barja G. 2004. Free radicals and aging. *Trends in Neurosciences* **27**:595–600. DOI: 10.1016/j.tins.2004.07.005

Barrows CH, Kokkonen GC. 2018. Dietary restriction and life extension—biological mechanisms. *Nutritional Approaches to Aging Research*. CRC Press. p. 219–244. See: https://www.taylorfrancis.com/chapters/edit/10.1201/9781351075121-12/dietary-restriction-life-extension-biological-mechanisms-charles-barrows-gertrude-kokkonen?utm_source=researchgate.net&utm_medium=article

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**:1–48. DOI: https://doi.org/10.18637/jss.v067.i01

Beintema JJ, Stam WT, Hazes B, Smidt MP. 1994. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Molecular Biology and Evolution* **11**:493–503. DOI: https://doi.org/10.1093/oxfordjournals.molbev.a040129

Bernadou A, Hoffacker E, Pable J, Heinze J. 2020. Lipid content influences division of labour in a clonal ant. *The Journal of Experimental Biology* **223**:jeb.219238. DOI: https://doi.org/10.1242/jeb.219238

Bernadou A, Schrader L, Pable J, Hoffacker E, Meusemann K, Heinze J. 2018. Stress and early experience underlie dominance status and division of labour in a clonal insect. *Proceedings of the Royal Society B: Biological Sciences* **285**:20181468. DOI: https://doi.org/10.1098/rspb.2018.1468

Bernstein KE, Ong FS, Blackwell W-LB, Shah KH, Giani JF, Gonzalez-Villalobos RA, Shen XZ, Fuchs S. 2013. A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacological Reviews* **65**:1. DOI: https://doi.org/10.1124/pr.112.006809

Beros S, Lenhart A, Scharf I, Negroni MA, Menzel F, Foitzik S. 2021. Extreme lifespan extension in tapeworm-infected ant workers. *Royal Society Open Science* **8**:202118. DOI: https://doi.org/10.1098/rsos.202118

Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signalling. *Nature reviews Molecular cell biology* **1**:11–21.

Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. 2010. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. *Cell metabolism* **11**:35–46.

References

Blacher P, Huggins TJ, Bourke AFG. 2017. Evolution of ageing, costs of reproduction and the fecundity–longevity trade-off in eusocial insects. *Proceedings of the Royal Society B: Biological Sciences* **284**:20170380. DOI: https://doi.org/10.1098/rspb.2017.0380

Blacher P, Lecoutey E, Fresneau D, Nowbahari E. 2010. Reproductive hierarchies and status discrimination in orphaned colonies of *Pachycondyla apicalis* ants. *Animal Behaviour* **79**:99–105. DOI: https://doi.org/10.1016/j.anbehav.2009.10.008

Blagosklonny MV. 2010. Why men age faster but reproduce longer than women: mTOR and evolutionary perspectives. *Aging* (Albany NY) **2**:265. DOI: 10.18632/aging.100149

Blagosklonny MV. 2008. Aging: ROS or TOR. *Cell cycle* **7**:3344–3354. DOI: 10.4161/cc.7.21.6965

Blanchard GB, Orledge GM, Reynolds SE, Franks NR. 2000. Division of labour and seasonality in the ant *Leptothorax albipennis*: worker corpulence and its influence on behaviour. *Animal Behaviour* **59**:723–738. DOI: https://doi.org/10.1006/anbe.1999.1374

Blum M, Chang HY, Chuguransky S, Grego T, Kandasaamy S, Mitchell A, Nuka G, Paysan-Lafosse T, Qureshi M, Raj S, Richardson L, Salazar GA, Williams L, Bork P, Bridge A, Gough J, Haft DH, Letunic I, Marchler-Bauer A, Mi H, Natale DA, Necci M, Orengo CA, Pandurangan AP, Rivoire C, Sigrist CJA, Sillitoe I, Thanki N, Thomas PD, Tosatto SCE, Wu CH, Bateman A, Finn RD. 2021. The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res* **49**:D344–D354. DOI: https://doi.org/10.1093/nar/gkaa977

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of lifespan by introduction of telomerase into normal human cells. *science* **279**:349–352. DOI: 10.1126/science.279.5349.349

Bogerd J, Babin PJ, Kooiman FP, André M, Ballagny C, Van Marrewijk WJA, Van Der Horst DJ. 2000. Molecular characterization and gene expression in the eye of the apolipophorin II/I precursor from *Locusta migratoria*. *Journal of Comparative Neurology* **427**:546–558. DOI: https://doi.org/10.1002/1096-9861(20001127)427:4%253C546::AID-CNE4%253E3.0.CO;2-H

References

Bonasio R, Li Q, Lian J, Mutti NS, Jin L, Zhao H, Zhang P, Wen P, Xiang H, Ding Y, Jin Z, Shen SS, Wang Z, Wang W, Wang J, Berger SL, Liebig J, Zhang G, Reinberg D. 2012. Genome-wide and caste-specific DNA methylomes of the ants *Camponotus floridanus* and *Harpegnathos saltator*. *Current Biology* **22**:1755–1764. DOI: https://doi.org/10.1016/j.cub.2012.07.042

Boomsma JJ. 2009. Lifetime monogamy and the evolution of eusociality. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**:3191–3207. DOI: https://doi.org/10.1098/rstb.2009.0101

Boomsma JJ, Gawne R. 2018. Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. *Biological Reviews* **93**:28–54. DOI: https://doi.org/10.1111/brv.12330

Boonstra J, Rijken P, Humbel B, Cremers F, Verkleij A, en Henegouwen P van B. 1995. The epidermal growth factor. *Cell Biology International* **19**:413–430. DOI: https://doi.org/10.1006/cbir.1995.1086

Boulay R, Lenoir A. 2001. Social isolation of mature workers affects nestmate recognition in the ant *Camponotus fellah*. *Behavioural Processes* **55**:67–73. DOI: https://doi.org/10.1016/S0376-6357(01)00163-2

Bourke AFG. 2011. Principles of social evolution. *Oxford University Press*. DOI: https://doi.org/10.1093/acprof:oso/9780199231157.001.0001

Bourke AFG. 1988. Worker reproduction in the higher eusocial Hymenoptera. *The Quarterly Review of Biology* **63**:291–311. See: https://www.jstor.org/stable/2830426

Braim BS. 2015. Exploring the regulatory role of behaviour and genome architecture in the socially polymorphic ant, *Leptothorax acervorum*. University of Leicester. Thesis. See: https://hdl.handle.net/2381/36076

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology* **34**:525–527. DOI: https://doi.org/10.1038/nbt.3519

Breed MD, Gamboa GJ. 1977. Behavioral control of workers by queens in primitively eusocial bees. *Science* **195**:694–696. DOI: https://doi.org/10.1126/science.195.4279.694

References

Brelsford A, Purcell J, Avril A, Tran Van P, Zhang J, Brütsch T, Sundström L, Helanterä H, Chapuisat M. 2020. An ancient and eroded social supergene Is widespread across Formica ants. *Current Biology* **30**:304-311.e4. DOI: https://doi.org/10.1016/j.cub.2019.11.032

Brunner E, Heinze J. 2009. Worker dominance and policing in the ant *Temnothorax unifasciatus*. *Insectes Sociaux* **56**:397–404. DOI: https://doi.org/10.1007/s00040-009-0037-x

Brunner E, Kellner K, Heinze J. 2009. Policing and dominance behaviour in the parthenogenetic ant *Platythyrea punctata*. *Animal Behaviour* **78**:1427–1431. DOI: https://doi.org/10.1016/j.anbehav.2009.09.022

Buschinger A. 1978. Genetisch bedingte Entstehung geflügelter Weibchen bei der sklavenhaltenden Ameise *Harpagoxenus sublaevis* (Nyl.) (Hym., Form.). *Insectes Sociaux* **25**:163–172. DOI: https://doi.org/10.1007/BF02224255

Buschinger A, Schreiber M. 2002. Queen polymorphism and queen-morph related facultative polygyny in the ant, *Myrmecina graminicola* (Hymenoptera, Formicidae). *Insectes Sociaux* **49**:344–353. DOI: https://doi.org/10.1007/PL00012658

Calabi P, Traniello JF. 1989. Behavioral flexibility in age castes of the ant *Pheidole dentata*. *Journal of Insect Behavior* **2**:663–677. DOI: https://doi.org/10.1007/BF01065785

Carey JR. 2001. Demographic mechanisms for the evolution of long life in social insects. *Slowly Aging Organisms* **36**:713–722. DOI: https://doi.org/10.1016/S0531-5565(00)00237-0

Carmona-Aldana F, Yong LW, Reinberg D, Desplan C. 2024. Phenomenon of reproductive plasticity in ants. *Current Opinion in Insect Science* **63**:101197. DOI: https://doi.org/10.1016/j.cois.2024.101197

Cervoni MS, Cardoso-Júnior CAM, Craveiro G, Souza A de O, Alberici LC, Hartfelder K. 2017. Mitochondrial capacity, oxidative damage and hypoxia gene expression are associated with age-related division of labor in honey bee (*Apis mellifera* L.) workers. *Journal of Experimental Biology* **220**:4035–4046. DOI: https://doi.org/10.1242/jeb.161844

References

Chandra V, Fetter-Pruneda I, Oxley PR, Ritger AL, McKenzie SK, Libbrecht R, Kronauer DJC. 2018. Social regulation of insulin signaling and the evolution of eusociality in ants. Science **361**:398–402. DOI: https://doi.org/10.1126/science.aar5723

Chapuisat M, Keller L. 2002. Division of labour influences the rate of ageing in weaver ant workers. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **269**:909–913. DOI: https://doi.org/Pa

Chaudhari SN, Kipreos ET. 2018. The energy maintenance theory of aging: maintaining energy metabolism to allow longevity. *BioEssays* **40**:1800005. DOI: https://doi.org/10.1002/bies.201800005

Chen J, Cui D-N, Ullah H, Li S, Pan F, Xu C-M, Tu X-B, Zhang Z-H. 2020. The function of *LmPrx6* in diapause regulation in *Locusta migratoria* through the insulin signaling pathway. *Insects* **11**. DOI: https://doi.org/10.3390/insects11110763

Chen J, Nouzová M, Noriega FG, Tatar M. 2024. Gut-to-brain regulation of *Drosophila* aging through neuropeptide F, insulin, and juvenile hormone. *Proceedings of the National Academy of Sciences* **121**:e2411987121. DOI: https://doi.org/10.1073/pnas.2411987121

Chen S. 2023. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta* **2**:e107. DOI: https://doi.org/10.1002/imt2.107

Chen S, Zhou Y, Chen Y, Gu J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**:i884–i890. DOI: https://doi.org/10.1093/bioinformatics/bty560

Chen YP, Vinson SP. 2000. Effects of queen attractiveness to workers on the queen nutritional status and egg production in the polygynous *Solenopsis invicta* (Hymenoptera: Formicidae). *Annals of the Entomological Society of America* **93**:295–302. DOI: https://doi.org/10.1603/0013-8746(2000)093%255B0295:EOQATW%255D2.0.CO;2

Choppin M, Feldmeyer B, Foitzik S. 2021. Histone acetylation regulates the expression of genes involved in worker reproduction in the ant *Temnothorax rugatulus*. *BMC Genomics* **22**:871. DOI: https://doi.org/10.1186/s12864-021-08196-8

Clark TG, Bradburn MJ, Love SB, Altman DG. 2003. Survival analysis part I: basic concepts and first analyses. *British Journal of Cancer* **89**:232–238. DOI: https://doi.org/10.1038/sj.bjc.6601118

References

Clutton-Brock T. 2002. Breeding together: kin selection and mutualism in cooperative vertebrates. *Science* **296**:69–72. DOI: https://doi.org/10.1126/science.296.5565.69

Cohen AA. 2018. Aging across the tree of life: The importance of a comparative perspective for the use of animal models in aging. *Model Systems of Aging* **1864**:2680–2689. DOI: https://doi.org/10.1016/j.bbadis.2017.05.028

Coles JA, Tsacopoulos M. 1981. Ionic and possible metabolic interactions between sensory neurones and glial cells in the retina of the honeybee drone. *Journal of Experimental Biology* **95**:75–92. DOI: https://doi.org/10.1242/jeb.95.1.75

Colgan TJ, Fletcher IK, Arce AN, Gill RJ, Ramos Rodrigues A, Stolle E, Chittka L, Wurm Y. 2019. Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees. *Molecular Ecology* **28**:1964–1974. DOI: https://doi.org/10.1111/mec.15047

Conway JR. 1986. The biology of honey ants. *The American Biology Teacher* **48**:335–343. DOI: https://doi.org/10.2307/4448321

Cooper GA, West SA. 2018. Division of labour and the evolution of extreme specialization. *Nature Ecology & Evolution* **2**:1161–1167. DOI: https://doi.org/10.1038/s41559-018-0564-9

Corona M, Libbrecht R, Wheeler DE. 2016. Molecular mechanisms of phenotypic plasticity in social insects. I*nsect genomics * Development and regulation* **13**:55–60. DOI: https://doi.org/10.1016/j.cois.2015.12.003

Corona M, Libbrecht R, Wurm Y, Riba-Grognuz O, Studer RA, Keller L. 2013. Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*. *PLOS Genetics* **9**:e1003730. DOI: https://doi.org/10.1371/journal.pgen.1003730

Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, Hughes KA, Robinson GE. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proceedings of the National Academy of Sciences* **104**:7128–7133. DOI: https://doi.org/10.1073/pnas.0701909104

Costa JT. 2006. The other insect societies. *Harvard University Press*.

Cousin M, Silva-Zacarin E, Kretzschmar A, El Maataoui M, Brunet J-L, Belzunces LP. 2013. Size changes in honey bee larvae oenocytes induced by exposure to paraquat at

very low concentrations. *PLOS ONE* **8**:e65693. DOI: https://doi.org/10.1371/journal.pone.0065693

Crespi B, Yanega D. 1995. The definition of eusociality. *Behavioral Ecology* **6**. DOI: https://doi.org/10.1093/beheco/6.1.109

Crozier RH, Pamilo P. 1996. Evolution of social insect colonies: sex allocation and kin selection. *Oxford University Press*.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G, Durbin R, 1000 Genomes project analysis group. 2011. The variant call format and VCFtools. *Bioinformatics* **27**:2156–2158. DOI: https://doi.org/10.1093/bioinformatics/btr330

Davidson NM, Hawkins ADK, Oshlack A. 2017. SuperTranscripts: a data driven reference for analysis and visualisation of transcriptomes. *Genome Biology* **18**:148. DOI: https://doi.org/10.1186/s13059-017-1284-1

De Gasperin O, Blacher P, Choppin M, Chapuisat M. 2025. Supergene regulation of ant social organization: a P haplotype in workers shifts colony ontogeny towards multiple queens. *Communications Biology* **8**:1035. DOI: https://doi.org/10.1038/s42003-025-08438-5

de Magalhães JP. 2024. Distinguishing between driver and passenger mechanisms of aging. *Nature Genetics* **56**:204–211. DOI: https://doi.org/10.1038/s41588-023-01627-0

de Magalhaes JP, Church GM. 2006. Cells discover fire: Employing reactive oxygen species in development and consequences for aging. **Experimental gerontology 41**:1–10. DOI: 10.1016/j.exger.2005.09.002

de Magalhães JP, Church GM. 2005. Genomes optimize reproduction: aging as a consequence of the developmental program. *Physiology* **20**:252–259. DOI: https://doi.org/10.1152/physiol.00010.2005

de Magalhaes JP, Wuttke D, Wood SH, Plank M, Vora C. 2012. Genome-environment interactions that modulate aging: powerful targets for drug discovery. *Pharmacological reviews* **64**:88–101. DOI: 10.1124/pr.110.004499

References

Dekoninck W, Parmentier T, Seifert B. 2016. First records of a supercolonial species of the *Tapinoma nigerrimum* complex in Belgium (Hymenoptera: Formicidae). *Bulletin de la Société royale belge d'Entomologie*.

D'Ettorre P, Heinze J, Schulz C, Francke W, Ayasse M. 2004. Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant P*achycondyla inversa*. *Journal of Experimental Biology* **207**:1085–1091. DOI: https://doi.org/10.1242/jeb.00865

Deveale B, Brummel T, Seroude L. 2004. Immunity and aging: The enemy within? *Aging cell* **3**:195–208. DOI: https://doi.org/10.1111/j.1474-9728.2004.00106.x

Dijkstra MB, Boomsma JJ. 2007. The economy of worker reproduction in *Acromyrmex* leafcutter ants. *Animal Behaviour* **74**:519–529. DOI: https://doi.org/10.1016/j.anbehav.2006.11.020

Dijkstra MB, Boomsma JJ. 2006. Are workers of *Atta* leafcutter ants capable of reproduction? *Insectes Sociaux* **53**:136–140. DOI: https://doi.org/10.1007/s00040-005-0848-3

Dijkstra MB, van Zweden JS, Dirchsen M, Boomsma JJ. 2010. Workers of *Acromyrmex echinatior* leafcutter ants police worker-laid eggs, but not reproductive workers. *Animal Behaviour* **80**:487–495. DOI: https://doi.org/10.1016/j.anbehav.2010.06.011

Dixon L, Kuster R, Rueppell O. 2014. Reproduction, social behavior, and aging trajectories in honeybee workers. *AGE* **36**:89–101. DOI: https://doi.org/10.1007/s11357-013-9546-7

Dubrovsky EB, Dubrovskaya VA, Berger EM. 2002. Juvenile hormone signaling during oogenesis in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology* **32**:1555–1565. DOI: https://doi.org/10.1016/S0965-1748(02)00076-0

Efeyan A, Comb WC, Sabatini DM. 2015. Nutrient-sensing mechanisms and pathways. *Nature* **517**:302–310. DOI: https://doi.org/10.1038/nature14190

Ekbote U, Coates D, Isaac RE. 1999. A mosquito (*Anopheles stephensi*) angiotensin I-converting enzyme (ACE) is induced by a blood meal and accumulates in the developing ovary. *FEBS Letters* **455**:219–222. DOI: https://doi.org/10.1016/S0014-5793(99)00870-4

References

Ekbote U, Looker M, Isaac RE. 2003a. ACE inhibitors reduce fecundity in the mosquito, *Anopheles stephensi*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **134**:593–598. DOI: https://doi.org/10.1016/S1096-4959(03)00019-8

Ekbote U, Weaver RJ, Isaac RE. 2003b. Angiotensin I-converting enzyme (ACE) activity of the tomato moth, *Lacanobia oleracea*: changes in levels of activity during development and after copulation suggest roles during metamorphosis and reproduction. I*nsect Biochemistry and Molecular Biology* **33**:989–998. DOI: https://doi.org/10.1016/S0965-1748(03)00105-X

Ekeoma BC, Ekeoma LN, Yusuf M, Haruna A, Ikeogu CK, Merican ZMA, Kamyab H, Pham CQ, Vo D-VN, Chelliapan S. 2023. Recent advances in the biocatalytic mitigation of emerging pollutants: A comprehensive review. *Journal of Biotechnology* **369**:14–34. DOI: https://doi.org/10.1016/j.jbiotec.2023.05.003

Elsner D, Meusemann K, Korb J. 2018. Longevity and transposon defense, the case of termite reproductives. *Proceedings of the National Academy of Sciences* **115**:5504–5509. DOI: https://doi.org/10.1073/pnas.1804046115

Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biology* **20**:238. DOI: https://doi.org/10.1186/s13059-019-1832-y

Errbii M, Ernst U, Lajmi A, Privman E, Gadau J, Schrader L. 2022. The *Pogonomyrmex californicus* social niche polymorphism is a polygenic trait involving a young supergene. *bioRxiv* **10**:21–436260. DOI: https://doi.org/10.1101/2021.03.21.436260

Evans J, Aronstein K, Chen YP, Hetru C, Imler J -L, Jiang H, Kanost M, Thompson G, Zou Z, Hultmark D. 2006. Immune pathways and defence mechanisms in honey bee *Apis mellifera*. *Insect Molecular Biology* **15**:645–656. DOI: https://doi.org/10.1111/j.1365-2583.2006.00682.x

Fabian DK, Fuentealba M, Dönertaş HM, Partridge L, Thornton JM. 2021. Functional conservation in genes and pathways linking ageing and immunity. *Immunity & Ageing* **18**:23. DOI: https://doi.org/10.1186/s12979-021-00232-1

References

Favreau E, Lebas C, Stolle E, Priyam A, Pracana R, Aron S, Wurm Y. 2022 No supergene despite social polymorphism in the big-headed ant *Pheidole pallidula*. *bioRxiv*, 2022.12.06.519286. DOI:10.1101/2022.12.06.519286)

Feldmeyer B, Elsner D, Foitzik S. 2014. Gene expression patterns associated with caste and reproductive status in ants: worker-specific genes are more derived than queen-specific ones. *Molecular Ecology* **23**:151–161. DOI: https://doi.org/10.1111/mec.12490

Fernanda Vergara-Martínez M, Otero-Díaz B, Fetter-Pruneda I. 2024. Reproductive Ageing: Unlocking the secrets of reproductive longevity: the potential of social insects. *Reproduction* **167**:e240020. DOI: https://doi.org/10.1530/REP-24-0020

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* **408**:239–247. DOI: https://doi.org/10.1038/35041687

Flatt T. 2011. Survival costs of reproduction in *Drosophila*. *Aging studies in Drosophila melanogaster* **46**:369–375. DOI: https://doi.org/10.1016/j.exger.2010.10.008

Flatt T, Kawecki TJ. 2007. Juvenile hormone as a regulator of the trade-off between reproduction and lifespan in *Drosophila melanogaster*. *Evolution* **61**:1980–1991. DOI: https://doi.org/10.1111/j.1558-5646.2007.00151.x

Fletcher DJC, Ross KG. 1985. Regulation of reproduction in eusocial hymenoptera. *Annual Review of Entomology*. DOI: https://doi.org/10.1146/annurev.en.30.010185.001535

Foitzik S, Fröba J, Rüger M, Witte V. 2011a. Competition over workers: fertility signalling in wingless queens of *Hypoponera opacior*. Insectes Sociaux **58**:271–278. DOI: https://doi.org/10.1007/s00040-011-0147-0

Foitzik S, Heinze J, Oberstadt B, Herbers JM. 2002. Mate guarding and alternative reproductive tactics in the ant *Hypoponera opacior*. Animal Behaviour **63**:597–604. DOI: https://doi.org/10.1006/anbe.2001.1945

Foitzik S, Kureck I, Rüger M, Metzler D. 2010. Alternative reproductive tactics and the impact of local competition on sex ratios in the ant *Hypoponera opacior*. *Behavioral Ecology and Sociobiology* **64**:1641–1654. DOI: https://doi.org/10.1007/s00265-010-0977-1

Foitzik S, Rüger MH, Kureck IM, Metzler D. 2011b. Macro- and microgeographic genetic structure in an ant species with alternative reproductive tactics in sexuals. J*ournal*

of Evolutionary Biology **24**:2721–2730. DOI: https://doi.org/10.1111/j.1420-9101.2011.02397.x

Fontana L, Partridge L, Longo VD. 2010. Extending healthy life span—from yeast to humans. *Science* **328**:321–326. DOI: https://doi.org/10.1126/science.1172539

Fox J, Weisberg S, Adler D, Bates D, Baud-Bovy G, Bolker B, Ellisom S, Firth D, Friendly M, Gorjanc G, Graves S, Heiberger R, Krivitsky P, Laboissiere R, Mächler M, Monette G, Murdoch D, Nilsson H, Ogle D, Ripley B, Short T, Venables W, Walker S, Winsemius D, Zeileis A. 2024. Companion to applied regression, package 'car.' See: https://cloud.r-project.org/web/packages/car/car.pdf

Friedman DA, Johnson BR, Linksvayer TA. 2020. Distributed physiology and the molecular basis of social life in eusocial insects. *Hormones and Behavior* **122**:104757. DOI: https://doi.org/10.1016/j.yhbeh.2020.104757

Garrison E, Marth G. 2012. Haplotype-based variant detection from short-read sequencing. *arXiv* preprint. DOI: https://doi.org/arXiv:1207.3907%2520%255Bq-bio.GN%255D

Gems D, de Magalhães JP. 2021. The hoverfly and the wasp: A critique of the hallmarks of aging as a paradigm. *Ageing Research Reviews* **70**:101407. DOI: https://doi.org/10.1016/j.arr.2021.101407

Gems D, Doonan R. 2009. Antioxidant defense and aging in *C. elegans*: is the oxidative damage theory of aging wrong? *Cell cycle* **8**:1681–1687.

Gems D, Partridge L. 2013. Genetics of longevity in model organisms: debates and paradigm shifts. *Annual Review of Physiology*. DOI: https://doi.org/10.1146/annurev-physiol-030212-183712

Gems D, Riddle DL. 1996. Longevity in *Caenorhabditis elegans* reduced by mating but not gamete production. *Nature* **379**:723–725. DOI: https://doi.org/10.1038/379723a0

Ghaninia M, Haight K, Berger SL, Reinberg D, Zwiebel LJ, Ray A, Liebig J. 2017. Chemosensory sensitivity reflects reproductive status in the ant *Harpegnathos saltator*. *Scientific Reports* **7**:3732. DOI: https://doi.org/10.1038/s41598-017-03964-7

Giannakou ME, Partridge L. 2007. Role of insulin-like signalling in *Drosophila* lifespan. *Trends in Biochemical Sciences* **32**:180–188. DOI: https://doi.org/10.1016/j.tibs.2007.02.007

References

Gillooly JF, Allen AP, West GB, Brown JH. 2005. The rate of DNA evolution: Effects of body size and temperature on the molecular clock. *Proceedings of the National Academy of Sciences* **102**:140–145. DOI: https://doi.org/10.1073/pnas.0407735101

Giraldo YM, Muscedere ML, Traniello JFA. 2021. Eusociality and senescence: neuroprotection and physiological resilience to aging in insect and mammalian systems. *Frontiers in Cell and Developmental Biology* Volume **9-**2021. DOI: 10.3389/fcell.2021.673172

Giraldo YM, Traniello JFA. 2014. Worker senescence and the sociobiology of aging in ants. *Behavioral Ecology and Sociobiology* **68**:1901–1919. DOI: https://doi.org/10.1007/s00265-014-1826-4

Glastad KM, Roessler J, Gospocic J, Bonasio R, Berger SL. 2023. Long ant life span is maintained by a unique heat shock factor. *Genes & Development* **37**:398–417. DOI: https://doi.org/10.1101/gad.350250.122

Gobin B, Ito F. 2000. Queens and major workers of *Acanthomyrmex ferox* redistribute nutrients with trophic eggs. *Naturwissenschaften* **87**:323–326. DOI: https://doi.org/10.1007/s001140050731

Gobin B, Peeters C, Billen J. 1998. Production of trophic eggs by virgin workers in the ponerine ant *Gnamptogenys menadensis*. *Physiological Entomology* **23**:329–336. DOI: https://doi.org/10.1046/j.1365-3032.1998.234102.x

Goodisman MAD, Ross KG. 1999. Queen recruitment in a multiple-queen population of the fire ant *Solenopsis invicta*. *Behavioral Ecology* **10**:428–435. DOI: https://doi.org/10.1093/beheco/10.4.428

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology* 29:644–652. DOI: 10.1038/nbt.1883

Grasso DA, Mori A, Le Moli F. 1998. Chemical communication during foraging in the harvesting ant *Messor capitatus* (Hymenoptera, Formicidae). *Insectes Sociaux* **45**:85–96. DOI: https://doi.org/10.1007/s000400050071

Grasso DA, Wenseleers T, Mori A, Le Moli F, Billen J. 2000. Thelytokous worker reproduction and lack of *Wolbachia* infection in the harvesting ant *Messor*

capitatus. *Ethology Ecology & Evolution* **12**:309–314. DOI: https://doi.org/10.1080/08927014.2000.9522803

Gutiérrez-Valencia J, Fracassetti M, Berdan EL, Bunikis I, Soler L, Dainat J, Kutschera VE, Losvik A, Désamoré A, Hughes PW, Foroozani A, Laenen B, Pesquet E, Abdelaziz M, Pettersson OV, Nystedt B, Brennan AC, Arroyo J, Slotte T. 2022. Genomic analyses of the *Linum distyly* supergene reveal convergent evolution at the molecular level. *Current Biology* **32**:4360-4371.e6. DOI: https://doi.org/10.1016/j.cub.2022.08.042

Gutiérrez-Valencia J, Hughes PW, Berdan EL, Slotte T. 2021. The genomic architecture and evolutionary fates of supergenes. *Genome Biology and Evolution* **13**:evab057. DOI: https://doi.org/10.1093/gbe/evab057

Ha E-M, Oh C-T, Bae Y, Lee W-J. 2005. A direct role for dual oxidase in *Drosophila* gut Immunity. *Science* (New York, N.Y.) **310**:847–50. DOI: https://doi.org/10.1126/science.1117311

Haas B, Papanicolaou A. 2017. TransDecoder. See: https://transdecoder. github. io.

Hagedorn H, Kunkel J. 1979. Vitellogenin and vitellin in insects. *Annual Review of Entomology* **24**:475–505. DOI: https://doi.org/10.1146/annurev.en.24.010179.002355

Haight KL, Liebig J. 2025. Age-resistant worker reproductive potential and effect of helpers on prolonged lifespan in an ant. *BMC Biology* **23**:198. DOI: https://doi.org/10.1186/s12915-025-02305-9

Hamidi R, de Biseau J-C, Quinet Y. 2023. Worker reproduction in the highly polygynous ant *Crematogaster pygmaea* Forel, 1904 (Hymenoptera: Formicidae). *Sociobiology* **70**:e7903. DOI: https://doi.org/10.13102/sociobiology.v70i3.7903

Hamilton WD. 1964. The genetical evolution of social behaviour. I. *Journal of Theoretical Biology* **7**:1–16. DOI: https://doi.org/10.1016/0022-5193(64)90038-4

Hammond RL, Keller L. 2004. Conflict over male parentage in social insects. *PLoS Biol* **2**:E248. DOI: https://doi.org/10.1371/journal.pbio.0020248

Hannonen M, Sledge MF, Turillazzi S, Sundström L. 2002. Queen reproduction, chemical signalling and worker behaviour in polygyne colonies of the ant *Formica fusca*. *Animal Behaviour* **64**:477–485. DOI: https://doi.org/10.1006/anbe.2002.4001

References

Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M, Kenyon C. 2008. A role for autophagy in the extension of lifespan by dietary restriction in *C. elegans*. *PLOS Genetics* **4**:e24. DOI: https://doi.org/10.1371/journal.pgen.0040024

Harman D. 1956. Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology* **11**:298–300. DOI: https://doi.org/10.1093/geronj/11.3.298

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS. 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**:392–395. DOI: 10.1038/nature08221

Hartfelder K, Engels W. 1998. 2 Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. In: Pedersen RA, Schatten GP (Eds). *Current Topics in Developmental Biology*. Academic Press. p. 45–77. DOI: https://doi.org/10.1016/S0070-2153(08)60364-6

Hartmann A, Heinze J. 2003. Lay eggs, live longer: division of labor and life span in a clonal species. *Evolution* **57**:2424–2429. DOI: https://doi.org/10.1111/j.0014-3820.2003.tb00254.x

Hartmann C, Haschlar J, Heinze J, Bernadou A. 2020. Activity patterns and age-dependent changes in behavior in the clonal ant *Platythyrea punctata*. *Journal of Insect Behavior* **33**:149–157. DOI: https://doi.org/10.1007/s10905-020-09756-8

Hartmann C, Heinze J, Bernadou A. 2019. Age-dependent changes in cuticular color and pteridine levels in a clonal ant. *Journal of Insect Physiology* **118**:103943. DOI: https://doi.org/10.1016/j.jinsphys.2019.103943

Hayflick L, Moorhead PS. 1961. The serial cultivation of human diploid cell strains. *Experimental cell research* **25**:585–621.

Hazel JR, Eugene Williams E. 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research* **29**:167–227. DOI: https://doi.org/10.1016/0163-7827(90)90002-3

Heine J, Buschinger A. 1989. Queen polymorphism in *Leptothorax spec. A:* Its genetic and ecological background (Hymenoptera: Formicidae). *Insectes Sociaux* **36**:139–155. DOI: https://doi.org/10.1007/BF02225909

References

Heinze J. 2017. Life-history evolution in ants: the case of *Cardiocondyla*. *Proceedings of the Royal Society B: Biological Sciences* **284**:20161406. DOI: https://doi.org/10.1098/rspb.2016.1406

Heinze J, Frohschammer S, Bernadou A. 2013. Queen life-span and total reproductive success are positively associated in the ant *Cardiocondyla cf. kagutsuchi*. *Behavioral Ecology and Sociobiology* **67**:1555–1562. DOI: https://doi.org/10.1007/s00265-013-1567-9

Heinze J, Giehr J. 2021. The plasticity of lifespan in social insects. *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190734. DOI: https://doi.org/10.1098/rstb.2019.0734

Heinze J, Puchinger W, Hölldobler B. 1997. Worker reproduction and social hierarchies in *Leptothorax* ants. *Animal Behaviour* **54**:849–864. DOI: https://doi.org/10.1006/anbe.1996.0511

Heinze J, Schrempf A. 2012. Terminal investment: individual reproduction of ant queens Increases with age. *PLOS ONE* **7**:e35201. DOI: https://doi.org/10.1371/journal.pone.0035201

Heinze J, Schrempf A. 2008. Aging and reproduction in social insects – A Mini-Review. *Gerontology* **54**:160–167. DOI: https://doi.org/10.1159/000122472

Heinze J, Tsuji K. 1995. Ant reproductive strategies. *Population Ecology* **37**:135–149. DOI: https://doi.org/10.1007/BF02515814

Helft F, Tirard C, Doums C. 2012. Effects of division of labour on immunity in workers of the ant *Cataglyphis cursor*. *Insectes Sociaux* **59**. DOI: https://doi.org/10.1007/s00040-012-0225-y

Helmkampf M, Cash E, Gadau J. 2015. Evolution of the insect desaturase gene family with an emphasis on social Hymenoptera. *Molecular Biology and Evolution* **32**:456–471. DOI: https://doi.org/10.1093/molbev/msu315

Herman W, Tatar M. 2002. Juvenile hormone regulation of aging in the migratory monarch butterfly. *Proceedings*. *Biological sciences / The Royal Society* **268**:2509–14. DOI: https://doi.org/10.1098/rspb.2001.1765

Hipp MS, Kasturi P, Hartl FU. 2019. The proteostasis network and its decline in ageing. *Nature Reviews Molecular Cell Biology* **20**:421–435. DOI: https://doi.org/10.1038/s41580-019-0101-y

References

Hodkova M. 2008. Tissue signaling pathways in the regulation of life-span and reproduction in females of the linden bug, *Pyrrhocoris apterus*. *Journal of Insect Physiology* **54**:508–517. DOI: https://doi.org/10.1016/j.jinsphys.2007.11.011

Hölldobler B, Wilson EO. 1990. “The Ants” Springer, Berlin, 732 pp. DM 198.—. J*ournal of Evolutionary Biology* **5**:169–171. DOI: https://doi.org/10.1046/j.1420-9101.1992.5010169.x

Hölldobler B, Wilson EO. 1983. Queen control in colonies of weaver ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America* **76**:235–238. DOI: https://doi.org/10.1093/aesa/76.2.235

Hölldobler B, Wilson EO. 1977. The number of queens: an important trait in ant evolution. *Naturwissenschaften* **64**:8–15. DOI: https://doi.org/10.1007/BF00439886

Holliday R. 1989. Food, reproduction and longevity: Is the extended lifespan of calorie-restricted animals an evolutionary adaptation? *Bioessays* 10:125–127. DOI: 10.1002/bies.950100408

Holman L. 2018. Queen pheromones and reproductive division of labor: A meta-analysis. *Behavioral Ecology* **29**:1199–1209. DOI: https://doi.org/10.1093/beheco/ary023

Holman L, Jørgensen C, Nielsen J. 2010. Identification of ant queen pheromone regulating worker sterility. *Proceedings. Biological sciences / The Royal Society* **277**:3793–800. DOI: https://doi.org/10.1098/rspb.2010.0984

Huang J, Wang S, Yu C, Su H, Jiang Z, Li X, Lu Y, Zhang J. 2025. Multi-omics analysis reveals TO gene’s Association with food selection and lifespan in minor-worker ants post-queen loss. *Ecology and Evolution* **15**:e71508. DOI: https://doi.org/10.1002/ece3.71508

Hulbert AJ, Clancy DJ, Mair W, Braeckman BP, Gems D, Partridge L. 2004. Metabolic rate is not reduced by dietary-restriction or by lowered insulin/IGF-1 signalling and is not correlated with individual lifespan in *Drosophila melanogaster*. *Experimental Gerontology* **39**:1137–1143. DOI: https://doi.org/10.1016/j.exger.2004.04.006

Hung CM, Garcia-Haro L, Sparks CA, Guertin DA. 2012. mTOR-dependent cell survival mechanisms. *Cold Spring Harbor Perspectives in Biology* **4**:a008771–a008771. DOI: https://doi.org/10.1101/cshperspect.a008771

Ihle KE, Mutti NS, Kaftanoglu O, Amdam GV. 2019. Insulin receptor substrate gene knockdown accelerates behavioural maturation and shortens lifespan in

honeybee workers. *Insects* **10**:390. DOI: https://doi.org/10.3390/insects10110390

Isaac RE, Ekbote U, Coates D, Shirras AD. 1999. Insect angiotensin-converting enzyme: a processing enzyme with broad substrate specificity and a role in reproduction. *Annals of the New York Academy of Sciences* **897**:342–347. DOI: https://doi.org/10.1111/j.1749-6632.1999.tb07904.x

Iwasa Y, Yamaguchi S. 2020. Task allocation in a cooperative society: specialized castes or age-dependent switching among ant workers. *Scientific Reports* **10**:3339. DOI: https://doi.org/10.1038/s41598-020-59920-5

Jaimes-Niño LM, Heinze J, Oettler J. 2022. Late-life fitness gains and reproductive death in *Cardiocondyla obscurior* ants. *eLife* **11**. DOI: https://doi.org/10.7554/eLife.74695

Janet C. 1907. Anatomie du corselet et histolyse des muscles vibrateurs, après le vol nuptial, chez la reine de la fourmi (*Lasius niger*). *Ducourtieux et Gout*.

Jehan C, Sabarly C, Rigaud T, Moret Y. 2022. Senescence of the immune defences and reproductive trade-offs in females of the mealworm beetle, *Tenebrio molitor*. *Scientific Reports* **12**:19747. DOI: https://doi.org/10.1038/s41598-022-24334-y

Jemielity S, Chapuisat M, Parker JD, Keller L. 2005. Long live the queen: studying aging in social insects. *AGE* **27**:241–248. DOI: https://doi.org/10.1007/s11357-005-2916-z

Jemielity S, Kimura M, Parker KM, Parker JD, Cao X, Aviv A, Keller L. 2007. Short telomeres in short-lived males: what are the molecular and evolutionary causes? *Aging Cell* **6**:225–233. DOI: https://doi.org/10.1111/j.1474-9726.2007.00279.x

Johnson SC, Rabinovitch PS, Kaeberlein M. 2013. mTOR is a key modulator of ageing and age-related disease. *Nature* **493**:338–345. DOI: https://doi.org/10.1038/nature11861

Jones OR, Scheuerlein A, Salguero-Gómez R, Camarda CG, Schaible R, Casper BB, Dahlgren JP, Ehrlén J, García MB, Menges ES, Quintana-Ascencio PF, Caswell H, Baudisch A, Vaupel JW. 2014. Diversity of ageing across the tree of life. *Nature* **505**:169–173. DOI: https://doi.org/10.1038/nature12789

Kandel ER. 2009. The biology of memory: a forty-year perspective. *The Journal of Neuroscience* **29**:12748–12756. DOI: https://doi.org/10.1523/JNEUROSCI.3958-09.2009

References

Kanost MR. 1999. Serine proteinase inhibitors in arthropod immunity. *Developmental & Comparative Immunology* **23**:291–301. DOI: https://doi.org/10.1016/S0145-305X(99)00012-9

Kanost MR, Clarke TE. 2005. 4.7 - Proteases. In: Gilbert LI (Ed). *Comprehensive Molecular Insect Science*. Elsevier. p. 247–265. DOI: https://doi.org/10.1016/B0-44-451924-6/00057-0

Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. 2004. Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Current Biology* **14**:885–890. DOI: https://doi.org/10.1016/j.cub.2004.03.059

Kassambara A, Kosinski M, Biecek P. 2025. survminer: Drawing survival curves using "ggplot2". R package version 0.5.1. See: https://kassambara.r-universe.dev/survminer/survminer.pdf

Kay T, Helleu Q, Keller L. 2022. Iterative evolution of supergene-based social polymorphism in ants. *Philosophical Transactions of the Royal Society B: Biological Sciences* **377**:20210196. DOI: https://doi.org/10.1098/rstb.2021.0196

Keller L. 1998. Queen lifespan and colony characteristics in ants and termites [Review]. I*nsectes Sociaux* **45**:235–246. DOI: https://doi.org/10.1007/s000400050084

Keller L. 1995. Social life: the paradox of multiple-queen colonies. T*rends in Ecology & Evolution* **10**:355–360. DOI: https://doi.org/10.1016/S0169-5347(00)89133-8

Keller L. 1988. Evolutionary implications of polygyny in the Argentine ant, *Iridomyrmex humilis* (Mayr) (Hymenoptera: Formicidae): an experimental study. *Animal Behaviour* **36**:159–165. DOI: https://doi.org/10.1016/S0003-3472(88)80259-8

Keller L, Genoud M. 1997. Extraordinary lifespans in ants: A test of evolutionary theories of ageing. *Nature* **389**:958–960. DOI: https://doi.org/10.1038/40130

Keller L, Jemielity S. 2006. Social insects as a model to study the molecular basis of ageing. *Experimental Gerontology* **41**:553–556. DOI: https://doi.org/10.1016/j.exger.2006.04.002

Kennedy A, Herman J, Rueppell O. 2021. Reproductive activation in honeybee (*Apis mellifera*) workers protects against abiotic and biotic stress. *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190737. DOI: https://doi.org/10.1098/rstb.2019.0737

References

Kenyon C. 2005. The plasticity of aging: insights from long-lived mutants. *Cell* **120**:449–460. DOI: https://doi.org/10.1016/j.cell.2005.02.002

Kenyon CJ. 2010. The genetics of ageing. *Nature* **464**:504–512. DOI: https://doi.org/10.1038/nature08980

Kim SY, Noguera JC, Morales J, Velando A. 2010. Heritability of resistance to oxidative stress in early life. *Journal of Evolutionary Biology* **23**:769–775. DOI: https://doi.org/10.1111/j.1420-9101.2010.01942.x

Kim YS, Nam HJ, Chung HY, Kim ND, Ryu JH, Lee WJ, Arking R, Yoo MA. 2001. Role of *xanthine dehydrogenase* and aging on the innate immune response of *Drosophila*. *Journal of the American Aging Association* **24**:187–193. DOI: https://doi.org/10.1007/s11357-001-0020-6

Kirkwood TBL. 2017. The disposable soma theory: origins and evolution. *The Evolution of Senescence in the Tree of Life*. p. 23–39. DOI: https://doi.org/10.1017/9781139939867.002

Kirkwood TBL, Austad SN. 2000. Why do we age? *Nature* **408**:233–238. DOI: https://doi.org/10.1038/35041682

Kirkwood TBL. 1977. Evolution of ageing. *Nature* **270**:301–304. DOI: https://doi.org/10.1038/270301a0

Kirkwood TBL, Holliday R. 1997. The evolution of ageing and longevity. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **205**:531–546. DOI: https://doi.org/10.1098/rspb.1979.0083

Klug A. 2005. The discovery of zinc fingers and their practical applications in gene regulation: a personal account. In: Iuchi S, Kuldell N (Eds). *Zinc Finger Proteins: From Atomic Contact to Cellular Function*. Springer US. p. 1–6. DOI: https://doi.org/10.1007/0-387-27421-9_1

Knaus BJ, Grünwald NJ. 2016. VcfR: a package to manipulate and visualize VCF format data in R. *bioRxiv* 041277. DOI: https://doi.org/10.1101/041277

Kohlmeier P, Alleman AR, Libbrecht R, Foitzik S, Feldmeyer B. 2019. Gene expression is more strongly associated with behavioural specialization than with age or fertility in ant workers. *Molecular Ecology* **28**:658–670. DOI: https://doi.org/10.1111/mec.14971

References

Kohlmeier P, Feldmeyer B, Foitzik S. 2018. Vitellogenin-like A–associated shifts in social cue responsiveness regulate behavioral task specialization in an ant. *PLOS Biology* **16**:e2005747. DOI: https://doi.org/10.1371/journal.pbio.2005747

Kohlmeier P, Holländer K, Meunier J. 2016. Survival after pathogen exposure in group-living insects: Don't forget the stress of social isolation! *Journal of Evolutionary Biology* **29**. DOI: https://doi.org/10.1111/jeb.12916

Kohlmeier P, Negroni MA, Kever M, Emmling S, Stypa H, Feldmeyer B, Foitzik S. 2017. Intrinsic worker mortality depends on behavioral caste and the queens' presence in a social insect. *The Science of Nature* **104**:34. DOI: https://doi.org/10.1007/s00114-017-1452-x

Kolora SRR, Owens GL, Vazquez JM, Stubbs A, Chatla K, Jainese C, Seeto K, McCrea M, Sandel MW, Vianna JA. 2021. Origins and evolution of extreme life span in Pacific Ocean rockfishes. Science **374**:842–847.

Konrad M, Pamminger T, Foitzik S. 2012. Two pathways ensuring social harmony. *Die Naturwissenschaften* **99**:627–36. DOI: https://doi.org/10.1007/s00114-012-0943-z

Korb J. 2016. Why do social insect queens live so long? Approaches to unravel the sociality-aging puzzle. *Vectors and medical and veterinary entomology * Social insects* **16**:104–107. DOI: https://doi.org/10.1016/j.cois.2016.06.004

Korb J, Heinze J. 2016. Major hurdles for the evolution of sociality. *Annual Review of Entomology* **61**:297–316. DOI: https://doi.org/10.1146/annurev-ento-010715-023711

Korb J, Meusemann K, Aumer D, Bernadou A, Elsner D, Feldmeyer B, Foitzik S, Heinze J, Libbrecht R, Lin S, Majoe M, Monroy Kuhn JM, Nehring V, Negroni MA, Paxton RJ, Séguret AC, Stoldt M, Flatt T, the So-Long consortium. 2021. Comparative transcriptomic analysis of the mechanisms underpinning ageing and fecundity in social insects. *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190728. DOI: https://doi.org/10.1098/rstb.2019.0728

Koto A, Tamura M, Wong PS, Aburatani S, Privman E, Stoffel C, Crespi A, McKenzie SK, La Mendola C, Kay T, Keller L. 2023. Social isolation shortens lifespan through oxidative stress in ants. *Nature Communications* **14**:5493. DOI: https://doi.org/10.1038/s41467-023-41140-w

References

Kowalczyk A, Partha R, Clark NL, Chikina M. 2020. Pan-mammalian analysis of molecular constraints underlying extended lifespan. *eLife* 9:e51089. DOI: 10.7554/eLife.51089

Kramer BH, Nehring V, Buttstedt A, Heinze J, Korb J, Libbrecht R, Meusemann K, Paxton RJ, Séguret A, Schaub F, Bernadou A. 2021. Oxidative stress and senescence in social insects: a significant but inconsistent link? *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190732. DOI: https://doi.org/10.1098/rstb.2019.0732

Kramer BH, Schaible R. 2013. Colony size explains the lifespan differences between queens and workers in eusocial Hymenoptera. *Biological Journal of the Linnean Society* **109**:710–724. DOI: https://doi.org/10.1111/bij.12072

Kramer BH, Schaible R, Scheuerlein A. 2016. Worker lifespan is an adaptive trait during colony establishment in the long-lived ant *Lasius niger*. *Experimental Gerontology* **85**:18–23. DOI: https://doi.org/10.1016/j.exger.2016.09.008

Kramer BH, Schrempf A, Scheuerlein A, Heinze J. 2015. Ant colonies do not trade-off reproduction against maintenance. *PLOS ONE* **10**:e0137969. DOI: https://doi.org/10.1371/journal.pone.0137969

Kramer BH, van Doorn GS, Arani BMS, Pen I. 2022. Eusociality and the evolution of aging in superorganisms. *The American Naturalist* **200**:63–80. DOI: https://doi.org/10.1086/719666

Kronauer DJC. 2009. Recent advances in army ant biology (Hymenoptera: Formicidae). *Myrmecological News* **12**:51–65. DOI: https://doi.org/10.25849/myrmecol.news_012:051

Kronauer DJC, Libbrecht R. 2018. Back to the roots: the importance of using simple insect societies to understand the molecular basis of complex social life. V*ectors and medical and veterinary entomology * Social insects* **28**:33–39. DOI: https://doi.org/10.1016/j.cois.2018.03.009

Kronauer DJC, Pierce NE, Keller L. 2012. Asexual reproduction in introduced and native populations of the ant *Cerapachys biroi*. *Molecular Ecology* **21**:5221–5235. DOI: https://doi.org/10.1111/mec.12041

References

Kronauer DJC, Schöning C, d'Ettorre P, Boomsma JJ. 2009. Colony fusion and worker reproduction after queen loss in army ants. *Proceedings of the Royal Society B: Biological Sciences* **277**:755–763. DOI: https://doi.org/10.1098/rspb.2009.1591

Kronauer DJC, Tsuji K, Pierce NE, Keller L. 2013. Non–nest mate discrimination and clonal colony structure in the parthenogenetic ant *Cerapachys biroi*. *Behavioral Ecology* **24**:617–622. DOI: https://doi.org/10.1093/beheco/ars227

Kruer MC, Jepperson T, Dutta S, Steiner RD, Cottenie E, Sanford L, Merkens M, Russman BS, Blasco PA, Fan G, Pollock J, Green S, Woltjer RL, Mooney C, Kretzschmar D, Paisán-Ruiz C, Houlden H. 2013. Mutations in gamma adducin are associated with inherited cerebral palsy. *Annals of Neurology* **74**:805–814. DOI: https://doi.org/10.1002/ana.23971

Kuhn TS, Hacking I. 1970. The structure of scientific revolutions. *University of Chicago press* Chicago.

Kureck IM, Jongepier E, Nicolai B, Foitzik S. 2012. No inbreeding depression but increased sexual investment in highly inbred ant colonies. *Molecular Ecology* **21**:5613–5623. DOI: https://doi.org/10.1111/mec.12060

Kureck IM, Nicolai B, Foitzik S. 2013. Similar performance of diploid and haploid males in an ant species without inbreeding avoidance. *Ethology* **119**:360–367. DOI: https://doi.org/10.1111/eth.12073

Kuszewska K, Miler K, Rojek W, Woyciechowski M. 2017. Honeybee workers with higher reproductive potential live longer lives. *Experimental Gerontology* **98**:8–12. DOI: https://doi.org/10.1016/j.exger.2017.08.022

Lagunas-Robles G, Purcell J, Brelsford A. 2021. Linked supergenes underlie split sex ratio and social organization in an ant. *Proceedings of the National Academy of Sciences* **118**:e2101427118. DOI: https://doi.org/10.1073/pnas.2101427118

Lajmi A, Cohen P, Lee C-C, Frenkel Z, Pellen Y, Privman E. 2025. Repeated evolution of supergenes on an ancient social chromosome. *bioRxiv* 2024.12.01.626239. DOI: https://doi.org/10.1101/2024.12.01.626239

LeBoeuf AC, Waridel P, Brent CS, Gonçalves AN, Menin L, Ortiz D, Riba-Grognuz O, Koto A, Soares ZG, Privman E, Miska EA, Benton R, Keller L. 2016. Oral transfer of chemical cues, growth proteins and hormones in social insects. *eLife* **5**:e20375. DOI: https://doi.org/10.7554/eLife.20375

References

Lee CC, Nakao H, Tseng SP, Hsu HW, Lin GL, Tay JW, Billen J, Ito F, Lee CY, Lin CC. 2017. Worker reproduction of the invasive yellow crazy ant *Anoplolepis gracilipes*. *Frontiers in Zoology* **14**:24.

Lee M, Yoon CS, Yi J, Cho JR, Kim HS. 2005. Cellular immune responses and FAD-glucose dehydrogenase activity of *Mamestra brassicae* (Lepidoptera: Noctuidae) challenged with three species of entomopathogenic fungi. *Physiological Entomology* **30**:287–292. DOI: https://doi.org/10.1111/j.1365-3032.2005.00460.x

Lenhart A, Majoe M, Selvi S, Colgan TJ, Libbrecht R, Foitzik S. 2025. Worker survival and egg production—but not transcriptional activity—respond to queen number in the highly polygynous, invasive ant *Tapinoma magnum*. *Molecular Ecology* **34(**6):e17679. DOI: https://doi.org/10.1111/mec.17679

Lenoir A, Cuisset D, Hefetz A. 2001. Effects of social isolation on hydrocarbon pattern and nestmate recognition in the ant *Aphaenogaster senilis* (Hymenoptera, Formicidae). *Insectes Sociaux* **48**:101–109. DOI: https://doi.org/10.1007/PL00001751

Lenoir A, Querard L, Pondicq N, Berton F. 1988. Reproduction and dispersal in the ant *Cataglyphis cursor* (Hymenoptera, Formicidae). *Psyche: A Journal of Entomology* **95**:054685. DOI: https://doi.org/10.1155/1988/54685

Lenth R. 2024. emmeans: Estimated marginal means, aka least-squares means. R package "emmeans." See: https://rvlenth.github.io/emmeans/

Li B, Bickel RD, Parker BJ, Saleh Ziabari O, Liu F, Vellichirammal NN, Simon JC, Stern DL, Brisson JA. 2020. A large genomic insertion containing a duplicated follistatin gene is linked to the pea aphid male wing dimorphism. *eLife* **9**:e50608. DOI: https://doi.org/10.7554/eLife.50608

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**:323. DOI: https://doi.org/10.1186/1471-2105-12-323

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**:1754–1760. DOI: https://doi.org/10.1093/bioinformatics/btp324

Li K, Jia QQ, Li S. 2019. Juvenile hormone signaling – a mini review. *Insect Science* **26**:600–606. DOI: https://doi.org/10.1111/1744-7917.12614

References

Li S, Vazquez JM, Sudmant PH. 2023. The evolution of aging and lifespan. *Trends in Genetics* **39**:830–843. DOI: https://doi.org/10.1016/j.tig.2023.08.005

Libbrecht R, Corona M, Wende F, Azevedo DO, Serrão JE, Keller L. 2013. Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proceedings of the National Academy of Sciences* **110**:11050–11055.

Libbrecht R, Oxley PR, Keller L, Kronauer DJC. 2016. Robust DNA methylation in the clonal raider ant brain. *Current Biology* **26**:391–395. DOI: https://doi.org/10.1016/j.cub.2015.12.040

Libbrecht R, Oxley PR, Kronauer DJC. 2018. Clonal raider ant brain transcriptomics identifies candidate molecular mechanisms for reproductive division of labor. *BMC Biology* **16**:89. DOI: https://doi.org/10.1186/s12915-018-0558-8

Libert S, Chao Y, Chu X, Pletcher SD. 2006. Trade-offs between longevity and pathogen resistance in *Drosophila melanogaster* are mediated by NFκB signaling. *Aging cell* **5**:533–543.

Lin S, Werle J, Korb J. 2021. Transcriptomic analyses of the termite, *Cryptotermes secundus*, reveal a gene network underlying a long lifespan and high fecundity. *Communications Biology* **4**:384. DOI: https://doi.org/10.1038/s42003-021-01892-x

Lochmiller R, Deerenberg C. 2000. Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos* **88**:87–98. DOI: https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lopes B, Campbell A, Contrera F. 2020. Queen loss changes behavior and increases longevity in a stingless bee. *Behavioral Ecology and Sociobiology* **74**. DOI: https://doi.org/10.1007/s00265-020-2811-8

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013. The hallmarks of aging. *Cell* **153**:1194–1217. DOI: https://doi.org/10.1016/j.cell.2013.05.039

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**:550. DOI: https://doi.org/10.1186/s13059-014-0550-8

References

Lucas ER, Keller L. 2014. Ageing and somatic maintenance in social insects. *Social insects/Global change biology* **5**:31–36. DOI: https://doi.org/10.1016/j.cois.2014.09.009

Ma S, Gladyshev VN. 2017. Molecular signatures of longevity: Insights from cross-species comparative studies. Presented at the Seminars in *Cell & Developmental Biology*. Elsevier. p. 190–203.

Madeo F, Zimmermann A, Maiuri MC, Kroemer G. 2015. Essential role for autophagy in life span extension. *The Journal of Clinical Investigation* **125**:85–93. DOI: https://doi.org/10.1172/JCI73946

Madgwick PG, Stewart B, Belcher LJ, Thompson CRL, Wolf JB. 2018. Strategic investment explains patterns of cooperation and cheating in a microbe. *Proceedings of the National Academy of Sciences* **115**:E4823–E4832. DOI: https://doi.org/10.1073/pnas.1716087115

Maegawa S, Hinkal G, Kim HS, Shen L, Zhang L, Zhang J, Zhang N, Liang S, Donehower LA, Issa J-PJ. 2010. Widespread and tissue specific age-related DNA methylation changes in mice. *Genome research* **20**:332–340.

Majoe M, Libbrecht R, Foitzik S, Nehring V. 2021. Queen loss increases worker survival in leaf-cutting ants under paraquat-induced oxidative stress. *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190735. DOI: https://doi.org/10.1098/rstb.2019.0735

Majoe M, Stolarek N, Vizueta J, Xiong Z, Schrader L, Boomsma JJ, Foitzik S, Libbrecht R, Nehring V. 2024. Queen loss fails to elicit physiological and transcriptional responses in workers of the invasive garden ant *Lasius neglectus*. *bioRxiv*. DOI: https://doi.org/10.1101/2024.01.26.577224

Maklakov AA, Chapman T. 2019. Evolution of ageing as a tangle of trade-offs: energy versus function. *Proceedings of the Royal Society B: Biological Sciences* **286**:20191604. DOI: https://doi.org/10.1098/rspb.2019.1604

Mandilaras K, Pathmanathan T, Missirlis F. 2013. Iron absorption in *Drosophila melanogaster*. *Nutrients* **5**:1622–1647. DOI: https://doi.org/10.3390/nu5051622

Martinez T, Wheeler DE. 1994. Storage proteins in adult ants (*Camponotus festinatus*): Roles in colony founding by queens and in larval rearing by workers. *Journal of*

Insect Physiology **40**:723–729. DOI: https://doi.org/10.1016/0022-1910(94)90100-7

Matte A, Billen J, Shit P, Heinze J, Bernadou A. 2024. Delayed maturation of the exoskeleton and muscle fibres in the ant *Platythyrea punctata*. *Biological Journal of the Linnean Society*. DOI: https://doi.org/10.1093/biolinnean/blae025

McCay CM, Crowell MF, Maynard LA. 1935. The effect of retarded growth upon the length of life Span and upon the ultimate body size: one figure. *The Journal of Nutrition* **10**:63–79. DOI: https://doi.org/10.1093/jn/10.1.63

Medawar PB. 1952. An unsolved problem of biology: an inaugural lecture delivered at *University College, London*, 6 December, 1951. H.K. Lewis and Company.

Merzendorfer H, Zimoch L. 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *Journal of Experimental Biology* **206**:4393–4412. DOI: https://doi.org/10.1242/jeb.00709

Min KT, Benzer S. 1997. Spongecake and eggroll: two hereditary diseases in *Drosophila* resemble patterns of human brain degeneration. *Current Biology* **7**:885–888. DOI: https://doi.org/10.1016/S0960-9822(06)00378-2

Miyazaki M, Ntambi JM. 2003. Role of stearoyl-coenzyme A desaturase in lipid metabolism. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **68**:113–121. DOI: https://doi.org/10.1016/S0952-3278(02)00261-2

Mockett RJ, Sohal RS. 2006. Temperature-dependent trade-offs between longevity and fertility in the *Drosophila* mutant, methuselah. *Experimental Gerontology* **41**:566–573. DOI: https://doi.org/10.1016/j.exger.2006.03.015

Moczek AP, Sultan S, Foster S, Ledón-Rettig C, Dworkin I, Nijhout HF, Abouheif E, Pfennig DW. 2011. The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B: Biological Sciences* **278**:2705–2713. DOI: https://doi.org/10.1098/rspb.2011.0971

Mona S, Gay EJ, Taupenot A, Ducancel J, Laso-Jadart R, Helleu Q, Chifflet-Belle P, Teodori E, Aury JM, Xiong Z, Schrader L, Vizueta J, Molet M, Doums C. 2025. Genomic evidence of a complex supergene system linking dispersal to social polymorphism. *Current Biology* **35**:6155-6162.e5. DOI: https://doi.org/10.1016/j.cub.2025.10.065

References

Monroy Kuhn JM, Meusemann K, Korb J. 2021. Disentangling the aging gene expression network of termite queens. *BMC Genomics* **22**:339. DOI: https://doi.org/10.1186/s12864-021-07649-4

Monroy Kuhn JM, Meusemann K, Korb J. 2019. Long live the queen, the king and the commoner? Transcript expression differences between old and young in the termite *Cryptotermes secundus*. *PLOS ONE* **14**:e0210371. DOI: https://doi.org/10.1371/journal.pone.0210371

Moroń D, Witek M, Woyciechowski M. 2008. Division of labour among workers with different life expectancy in the ant *Myrmica scabrinodis*. *Animal Behaviour* **75**:345–350. DOI: https://doi.org/10.1016/j.anbehav.2007.06.005

Münch D, Amdam GV. 2010. The curious case of aging plasticity in honey bees. *Gothenburg Special Issue: Molecules of Life* **584**:2496–2503. DOI: https://doi.org/10.1016/j.febslet.2010.04.007

Münch D, Amdam GV, Wolschin F. 2008. Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Functional Ecology* **22**:407–421. DOI: https://doi.org/10.1111/j.1365-2435.2008.01419.x

Musselman LP, Cedden D, Güney G, Toprak U. 2025. Insect lipidomics: advances, applications, and physiological insights. *Springer International Publishing*. p. 1–31. DOI: https://doi.org/10.1007/5584_2025_878

Naim N, Amrit FRG, Ratnappan R, DelBuono N, Loose JA, Ghazi A. 2021. Cell nonautonomous roles of NHR-49 in promoting longevity and innate immunity. *Aging Cell* **20**:e13413. DOI: https://doi.org/10.1111/acel.13413

Negroni MA, Feldmeyer B, Foitzik S. 2021a. Experimental increase in fecundity causes upregulation of fecundity and body maintenance genes in the fat body of ant queens. *Biology Letters* **17**:20200909. DOI: https://doi.org/10.1098/rsbl.2020.0909

Negroni MA, Foitzik S, Feldmeyer B. 2019. Long-lived *Temnothorax* ant queens switch from investment in immunity to antioxidant production with age. *Scientific Reports* **9**:7270. DOI: https://doi.org/10.1038/s41598-019-43796-1

Negroni MA, Macit MN, Stoldt M, Feldmeyer B, Foitzik S. 2021b. Molecular regulation of lifespan extension in fertile ant workers. *Philosophical Transactions of the Royal*

Society B: Biological Sciences **376**:20190736. DOI: https://doi.org/10.1098/rstb.2019.0736

Negroni MA, Segers FHID, Vogelweith F, Foitzik S. 2020. Immune challenge reduces gut microbial diversity and triggers fertility-dependent gene expression changes in a social insect. *BMC Genomics* **21**:816. DOI: https://doi.org/10.1186/s12864-020-07191-9

Negroni MA, Stoldt M, Oster M, Rupp AS, Feldmeyer B, Foitzik S. 2021c. Social organization and the evolution of life-history traits in two queen morphs of the ant *Temnothorax rugatulus*. *Journal of Experimental Biology* **224**:jeb232793. DOI: https://doi.org/10.1242/jeb.232793

Nilsen KA, Ihle KE, Frederick K, Fondrk MK, Smedal B, Hartfelder K, Amdam GV. 2011. Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. *Journal of Experimental Biology* **214**:1488–1497. DOI: https://doi.org/10.1242/jeb.050393

Nojima Y, Ito K, Ono H, Nakazato T, Bono H, Yokoyama T, Sato R, Suetsugu Y, Nakamura Y, Yamamoto K, Satoh J, Tabunoki H, Fugo H. 2015. Superoxide dismutases, SOD1 and SOD2, play a distinct role in the fat body during pupation in silkworm *Bombyx mori*. *PLOS ONE* **10**:e0116007. DOI: https://doi.org/10.1371/journal.pone.0116007

Oettler J, Schrempf A. 2016. Fitness and aging in *Cardiocondyla obscurior* ant queens. *Vectors and medical and veterinary entomology * Social insects* **16**:58–63. DOI: https://doi.org/10.1016/j.cois.2016.05.010

Oliveira RC, Warson J, Sillam-Dussès D, Herrera-Malaver B, Verstrepen K, Millar JG, Wenseleers T. 2020. Identification of a queen pheromone mediating the rearing of adult sexuals in the pharaoh ant *Monomorium pharaonis*. *Biology Letters* **16**:20200348. DOI: https://doi.org/10.1098/rsbl.2020.0348

Oliveros JC. 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. See: http://bioinfogp. cnb. csic. es/tools/venny/index. html.

Olovnikov AM. 1996. Telomeres, telomerase, and aging: origin of the theory. *Experimental gerontology* **31**:443–448.

References

Omholt SW, Amdam GV. 2004. Epigenetic regulation of aging in honeybee workers. *Science of Aging Knowledge Environment* **2004**:pe28–pe28. DOI: https://doi.org/10.1126/sageke.2004.26.pe28

Opachaloemphan C, Mancini G, Konstantinides N, Parikh A, Mlejnek J, Yan H, Reinberg D, Desplan C. 2021. Early behavioral and molecular events leading to caste switching in the ant *Harpegnathos*. *Genes & Development* **35**:410–424. DOI: https://doi.org/10.1101/gad.343699.120

Otarigho B, Aballay A. 2021. Immunity-longevity tradeoff neurally controlled by GABAergic transcription factor PITX1/UNC-30. *Cell Reports* **35**:109187. DOI: https://doi.org/10.1016/j.celrep.2021.109187

Oxley PR, Ji L, Fetter-Pruneda I, McKenzie SK, Li C, Hu H, Zhang G, Kronauer DJC. 2014. The genome of the clonal raider ant *Cerapachys biroi*. *Current Biology* **24**:451–458. DOI: https://doi.org/10.1016/j.cub.2014.01.018

Page RE, Peng CYS. 2001. Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Slowly Aging Organisms* **36**:695–711. DOI: https://doi.org/10.1016/S0531-5565(00)00236-9

Pal D, Raj K. 2022. Chapter 9 - Biological macromolecules acting on central nervous system. In: Nayak AK, Dhara AK, Pal D (Eds). *Biological Macromolecules*. Academic Press. p. 219–228. DOI: https://doi.org/10.1016/B978-0-323-85759-8.00009-9

Pantano L. 2017. DEGreport: Report of DEG analysis. DOI: https://doi.org/doi:10.18129/B9.bioc.DEGreport

Parker J. 2010. What are social insects telling us about aging? *Myrmecological News* **13**:103–110.

Partridge L, Gems D. 2002. Mechanisms of aging: public or private? *Nature Reviews Genetics* **3**:165–175. DOI: https://doi.org/10.1038/nrg753

Partridge L, Green A, Fowler K. 1987. Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *Journal of Insect Physiology* **33**:745–749. DOI: https://doi.org/10.1016/0022-1910(87)90060-6

Partridge L, Harvey PH. 1988. The ecological context of life history evolution. *Science* **241**:1449–1455. DOI: https://doi.org/10.1126/science.241.4872.1449

References

Pearcy M, Aron S, Doums C, Keller L. 2004. Conditional use of sex and parthenogenesis for worker and queen production in ants. *Science* **306**:1780–1783.

Pearcy M, Hardy O, Aron S. 2006. Thelytokous parthenogenesis and its consequences on inbreeding in an ant. *Heredity* **96**:377–382. DOI: https://doi.org/10.1038/sj.hdy.6800813

Pearl R. 1928. The rate of living: being an account of some experimental studies on the biology of life duration. See: https://archive.org/details/b29814832

Peeters C. 1993. Monogyny and polygyny in ponerine ants with or without queens. In Laurent Keller (ed.), *Queen Number and Sociality in Insects* (Oxford, 1993; online edn, Oxford Academic, 31 Oct. 2023).DOI: https://doi.org/10.1093/oso/9780198540571.003.0011

Peeters C. 1991. The occurrence of sexual reproduction among ant workers. *Biological Journal of The Linnean Society* **44**:141–152. DOI: https://doi.org/10.1111/j.1095-8312.1991.tb00612.x

Peeters C, Ito F. 2015. Wingless and dwarf workers underlie the ecological success of ants (Hymenoptera: Formicidae). *Myrmecological News* **21**:117–130. See: https://myrmecologicalnews.org/cms/index.php?option=com_download&view=download&filename=volume21/mn21_117-130_printable.pdf&format=raw

Peeters C, Liebig J, Hölldobler B. 2000. Sexual reproduction by both queens and workers in the ponerine ant *Harpegnathos saltator*. *Insectes Sociaux* **47**:325–332. DOI: https://doi.org/10.1007/PL00001724

Pentreath VW, Seal LH, Morrison JH, Magistretti PJ. 1986. Transmitter mediated regulation of energy metabolism in nervous tissue at the cellular level. N*eurochemistry International* **9**:1–10. DOI: https://doi.org/10.1016/0197-0186(86)90025-2

Pérez VI, Bokov A, Van Remmen H, Mele J, Ran Q, Ikeno Y, Richardson A. 2009. Is the oxidative stress theory of aging dead? *Biochimica et Biophysica Acta* (BBA)-General Subjects **1790**:1005–1014.

Pie M, Tschá M. 2013. Size and shape in the evolution of ant worker morphology. *PeerJ* **1**:e205. DOI: https://doi.org/10.7717/peerj.205

Pigliucci M. 2001. Phenotypic plasticity: beyond nature and nurture. *JHU* Press.

Porter SD. 1988. Efficiency of sperm use in queens of the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*

81:777–781. See: https://www.ars.usda.gov/ARSUserFiles/60360510/publications/Tschinkel_and_Porter-1988(M-2351).pdf

Powers RW, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. 2006. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes & Development* 20:174–184.DOI: 10.1101/gad.1381406

Pracana R, Priyam A, Levantis I, Nichols R, Wurm Y. 2017. The fire ant social chromosome supergene variant Sb shows low diversity but high divergence from SB. *Molecular ecology* **26**. DOI: https://doi.org/10.1111/mec.14054

Puig S, Ramos-Alonso L, Romero AM, Martínez-Pastor MT. 2017. The elemental role of iron in DNA synthesis and repair. *Metallomics* **9**:1483–1500. DOI: https://doi.org/10.1039/c7mt00116a

Purcell J, Brelsford A. 2025. Supergenes in organismal and social development of insects: ideas and opportunities. *Current Opinion in Insect Science* **68**:101303. DOI: https://doi.org/10.1016/j.cois.2024.101303

Purcell J, Brelsford A, Wurm Y, Perrin N, Chapuisat M. 2014. Convergent genetic architecture underlies social organization in ants. *Current Biology* **24**:2728–2732. DOI: https://doi.org/10.1016/j.cub.2014.09.071

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, Maller J, Sklar P, de Bakker PIW, Daly MJ, Sham PC. 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. *The American Journal of Human Genetics* **81**:559–575. DOI: https://doi.org/10.1086/519795

Qiu X, Huang W, Yue W, Li D, Zhi J. 2024. Response of the serine/threonine kinase AKT and phosphoinositide-dependent kinase PDK in *Frankliniella occidentalis* (Thysanoptera: Thripidae) to three kinds of foods and their regulation of reproductive function. *Insect Molecular Biology* **33**:372–386. DOI: https://doi.org/10.1111/imb.12905

Queller DC, Strassmann JE. 1998. Kin selection and social insects. *Bioscience* **48**:165–175.

Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**:841–842. DOI: https://doi.org/10.1093/bioinformatics/btq033

References

Quque M, Brun C, Villette C, Sueur C, Criscuolo F, Heintz D, Bertile F. 2023. Both age and social environment shape the phenotype of ant workers. *Scientific Reports* **13**:186. DOI: https://doi.org/10.1038/s41598-022-26515-1

Quque M, Villette C, Criscuolo F, Sueur C, Bertile F, Heintz D. 2021. Eusociality is linked to caste-specific differences in metabolism, immune system, and somatic maintenance-related processes in an ant species. *Cellular and Molecular Life Sciences* **79**:29. DOI: https://doi.org/10.1007/s00018-021-04024-0

R Core Team. 2024. R: A language and environment for statistical computing.

Rabeling C, Kronauer D. 2012. Thelytokous Parthenogenesis in eusocial Hymenoptera. *Annual review of entomology* **58**. DOI: https://doi.org/10.1146/annurev-ento-120811-153710

Ratnieks F, Foster K, Wenseleers T. 2006. Conflict resolution in insect societies. *Annual review of entomology* **51**:581–608. DOI: https://doi.org/10.1146/annurev.ento.51.110104.151003

Rau V, Korb J. 2021. The effect of environmental stress on ageing in a termite species with low social complexity. *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190739. DOI: https://doi.org/10.1098/rstb.2019.0739

Ravary F, Jahyny B, Jaisson P. 2006. Brood stimulation controls the phasic reproductive cycle of the parthenogenetic ant *Cerapachys biroi*. *Insectes Sociaux* **53**:20–26. DOI: https://doi.org/10.1007/s00040-005-0828-7

Ravary F, Jaisson P. 2004. Absence of individual sterility in thelytokous colonies of the ant *Cerapachys biroi* Forel (Formicidae, Cerapachyinae). I*nsectes Sociaux* **51**:67–73. DOI: https://doi.org/10.1007/s00040-003-0724-y

Ray PD, Huang B-W, Tsuji Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling* **24**:981–990. DOI: https://doi.org/10.1016/j.cellsig.2012.01.008

Retana J, Cerdá X. 1994. Worker size polymorphism conditioning size matching in two sympatric seed-harvesting ants. *Oikos* **71**:261–266. DOI: https://doi.org/10.2307/3546274

Reuter M, Balloux F, Lehmann L, Keller L. 2001. Kin structure and queen execution in the Argentine ant *Linepithema humile*. *Journal of Evolutionary Biology* **14**:954–958. DOI: https://doi.org/10.1046/j.1420-9101.2001.00345.x

References

Robinson EJ, Feinerman O, Franks NR. 2009. Flexible task allocation and the organization of work in ants. Proceedings of the Royal Society B: Biological Sciences 276:4373–4380. DOI: 10.1098/rspb.2009.1244

Rodrigues MA, Flatt T. 2016. Endocrine uncoupling of the trade-off between reproduction and somatic maintenance in eusocial insects. *Vectors and medical and veterinary entomology * Social insects* **16**:1–8. DOI: https://doi.org/10.1016/j.cois.2016.04.013

Rose MR. 1990. Evolutionary biology of aging. *Oxford University Press*. DOI: https://doi.org/10.1093/oso/9780195061338.001.0001

Ross KG, Keller L. 1995. Ecology and evolution of social organization: insights from fire ants and other highly eusocial insects. *Annual Review of Ecology and Systematics* **26**:631–656.

Rubinsztein DC, Mariño G, Kroemer G. 2011. Autophagy and aging. *Cell* **146**:682–695. DOI: https://doi.org/10.1016/j.cell.2011.07.030

Rueppell O, Christine S, Mulcrone C, Groves L. 2007. Aging without functional senescence in honey bee workers. *Current Biology* **17**:R274–R275. DOI: https://doi.org/10.1016/j.cub.2007.02.015

Scarparo G, Palanchon M, Brelsford A, Purcell J. 2023. Social antagonism facilitates supergene expansion in ants. *Current Biology* **33**:5085-5095.e4. DOI: https://doi.org/10.1016/j.cub.2023.10.049

Schilder K, Heinze J, Hölldobler B. 1999. Colony structure and reproduction in the thelytokous parthenogenetic ant *Platythyrea punctata* (F. Smith) (Hymenoptera, Formicidae). *Insectes Sociaux* **46**:150–158. DOI: https://doi.org/10.1007/s000400050126

Schlichting CD, Pigliucci M. 1998. Phenotypic evolution: a reaction norm perspective.

Schrempf A, Cremer S, Heinze J. 2011. Social influence on age and reproduction: reduced lifespan and fecundity in multi-queen ant colonies. *Journal of Evolutionary Biology* **24**:1455–1461. DOI: https://doi.org/10.1111/j.1420-9101.2011.02278.x

Schrempf A, Giehr J, Röhrl R, Steigleder S, Heinze J. 2017. Royal Darwinian demons: enforced changes in reproductive efforts do not affect the life expectancy of ant

queens. *The American Naturalist* **189**:436–442. DOI: https://doi.org/10.1086/691000

Schultner E, Oettler J, Helanterä H. 2017. The role of brood in eusocial Hymenoptera. T*he Quarterly Review of Biology* **92**:39–78.

Schwander T, Lo N, Beekman M, Oldroyd BP, Keller L. 2010. Nature versus nurture in social insect caste differentiation. *Trends in Ecology & Evolution* **25**:275–282. DOI: https://doi.org/10.1016/j.tree.2009.12.001

Seehuus SC, Norberg K, Gimsa U, Krekling T, Amdam GV. 2006. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. P*roceedings of the National Academy of Sciences* **103**:962–967. DOI: https://doi.org/10.1073/pnas.0502681103

Seid MA, Traniello JF. 2006. Age-related repertoire expansion and division of labor in *Pheidole dentata* (Hymenoptera: Formicidae): a new perspective on temporal polyethism and behavioral plasticity in ants. *Behavioral Ecology and Sociobiology* **60**:631–644.

Seifert B, D'Eustacchio D, Kaufmann B, Centorame M, Lorite P, Modica MV. 2017. Four species within the supercolonial ants of the *Tapinoma nigerrimum* complex revealed by integrative taxonomy (Hymenoptera: Formicidae). *Myrmecological News* **24**:123–144. DOI: https://doi.org/10.25849/myrmecol.news_024:123

Seistrup AS, Choppin M, Govind S, Feldmeyer B, Kever M, Karaulanov E, Séguret A, Karunanithi S, Almeida MV, Ketting RF, Foitzik S. 2023. Age- and caste-independent piRNAs in the germline and miRNA profiles linked to caste and fecundity in the ant *Temnothorax rugatulus*. *Molecular Ecology* **32**:6027–6043. DOI: https://doi.org/10.1111/mec.17162

Selman C, Blount JD, Nussey DH, Speakman JR. 2012. Oxidative damage, ageing, and life-history evolution: where now? *Trends in Ecology & Evolution* **27**:570–577. DOI: https://doi.org/10.1016/j.tree.2012.06.006

Sharma KR, Enzmann BL, Schmidt Y, Moore D, Jones GR, Parker J, Berger SL, Reinberg D, Zwiebel LJ, Breit B. 2015. Cuticular hydrocarbon pheromones for social behavior and their coding in the ant antenna. *Cell reports* **12**:1261–1271.

Sheng L, Shields EJ, Gospocic J, Glastad KM, Ratchasanmuang P, Berger SL, Raj A, Little S, Bonasio R. 2020. Social reprogramming in ants induces longevity-associated

glia remodeling. *Science Advances* **6**:eaba9869. DOI: https://doi.org/10.1126/sciadv.aba9869

Sheng Z, Xu J, Bai H, Zhu F, Palli SR. 2011. Juvenile hormone regulates Vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum**. *Journal of Biological Chemistry* **286**:41924–41936. DOI: https://doi.org/10.1074/jbc.M111.269845

Sieber KR, Dorman T, Newell N, Yan H. 2021. (Epi)genetic mechanisms underlying the evolutionary success of eusocial insects. *Insects* **12**. DOI: https://doi.org/10.3390/insects12060498

Sigeman H, Seppä P, Downing PA, Webster MT, Helanterä H, Viljakainen L. 2025. A novel supergene controls queen size and colony social organization in the ant *Myrmica ruginodis*. *Molecular Biology and Evolution* **42**:msaf255. DOI: https://doi.org/10.1093/molbev/msaf255

Simpson S, Raubenheimer D. 2009. Macronutrient balance and lifespan. *Aging* **1**:875–80. DOI: https://doi.org/10.18632/aging.100098

Simpson SJ, Sword GA, Lo N. 2011. Polyphenism in insects. *Current Biology* **21**:R738–R749. DOI: https://doi.org/10.1016/j.cub.2011.06.006

Singh PP, Demmitt BA, Nath RD, Brunet A. 2019. The genetics of aging: a vertebrate perspective. *Cell* **177**:200–220.

Sistermans T, Hartke J, Stoldt M, Libbrecht R, Foitzik S. 2023. The influence of parasite load on transcriptional activity and morphology of a cestode and its ant intermediate host. *Molecular Ecology* **32**:4412–4426. DOI: https://doi.org/10.1111/mec.16995

Sohal RS, Weindruch R. 1996. Oxidative stress, caloric restriction, and aging. *Science* **273**:59–63.

Soneson C, Love M, Robinson M D. 2016. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences [version 2; peer review: 2 approved]. **4**:1521. DOI: https://doi.org/10.12688/f1000research.7563.2

Speakman JR. 2005. Body size, energy metabolism and lifespan. *Journal of Experimental Biology* **208**:1717–1730. DOI: https://doi.org/10.1242/jeb.01556

References

Stacey DW. 2003. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Current Opinion in Cell Biology* **15**:158–163. DOI: https://doi.org/10.1016/S0955-0674(03)00008-5

Stanley D, Kim Y. 2019. Prostaglandins and other eicosanoids in insects: biosynthesis and biological actions. *Frontiers in Physiology* **9**. DOI: https://doi.org/10.3389/fphys.2018.01927

Stark G, Pincheira-Donoso D, Meiri S. 2020. No evidence for the 'rate-of-living' theory across the tetrapod tree of life. *Global Ecology and Biogeography* **29**:857–884. DOI: https://doi.org/10.1111/geb.13069

Strätz M, Heinze J. 2004. Colony structure and sex allocation ratios in the ant *Temnothorax crassispinus*. *Insectes Sociaux* **51**:372–377. DOI: https://doi.org/10.1007/s00040-004-0755-z

Stroeymeyt N, Brunner E, Heinze J. 2007. "Selfish worker policing" controls reproduction in a *Temnothorax* ant. *Behavioral Ecology and Sociobiology* **61**:1449–1457. DOI: https://doi.org/10.1007/s00265-007-0377-3

Sudyka J, Arct A, Drobniak SM, Gustafsson L, Cichoń M. 2019. Birds with high lifetime reproductive success experience increased telomere loss. *Biology Letters* **15**:20180637. DOI: https://doi.org/10.1098/rsbl.2018.0637

Szathmáry E, Smith JM. 1995. The major evolutionary transitions. *Nature* **374**:227–232. DOI: https://doi.org/10.1038/374227a0

Tain LS, Sehlke R, Meilenbrock RL, Leech T, Paulitz J, Chokkalingam M, Nagaraj N, Grönke S, Fröhlich J, Atanassov I, Mann M, Beyer A, Partridge L. 2021. Tissue-specific modulation of gene expression in response to lowered insulin signalling in *Drosophila*. *eLife* **10**:e67275. DOI: https://doi.org/10.7554/eLife.67275

Tarpy DR, Hatch S, Fletcher DJC. 2000. The influence of queen age and quality during queen replacement in honeybee colonies. *Animal Behaviour* **59**:97–101. DOI: https://doi.org/10.1006/anbe.1999.1311

Tasaki E, Kobayashi K, Matsuura K, Iuchi Y. 2018. Long-lived termite queens exhibit high Cu/Zn-Superoxide Dismutase activity. *Oxidative Medicine and Cellular Longevity* **2018**:1–8. DOI: https://doi.org/10.1155/2018/5127251

References

Tasaki E, Takata M, Matsuura K. 2021. Why and how do termite kings and queens live so long? *Philosophical Transactions of the Royal Society B: Biological Sciences* **376**:20190740. DOI: https://doi.org/10.1098/rstb.2019.0740

Teseo S, Kronauer DJC, Jaisson P, Châline N. 2013. Enforcement of reproductive synchrony via policing in a clonal ant. *Current Biology* **23**:328–332. DOI: https://doi.org/10.1016/j.cub.2013.01.011

Therneau T. 2024. Package 'coxme.' See: https://cran.csiro.au/web/packages/coxme/vignettes/coxme.pdf

Thompson MJ, Jiggins CD. 2014. Supergenes and their role in evolution. *Heredity* **113**:1–8. DOI: https://doi.org/10.1038/hdy.2014.20

Tian X, Seluanov A, Gorbunova V. 2017. Molecular mechanisms determining lifespan in short-and long-lived species. *Trends in Endocrinology & Metabolism* **28**:722–734.

Timmermans I, Hefetz A, Fournier D, Aron S. 2008. Population genetic structure, worker reproduction and thelytokous parthenogenesis in the desert ant *Cataglyphis sabulosa*. *Heredity* **101**:490–498.

Tissenbaum HA, Ruvkun G. 1998. An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans*. *Genetics* **148**:703–717. DOI: https://doi.org/10.1093/genetics/148.2.703

Tofilski A. 2002. Influence of age polyethism on longevity of workers in social insects. *Behavioral Ecology and Sociobiology* **51**:234–237. DOI: https://doi.org/10.1007/s00265-001-0429-z

Toivonen JM, Partridge L. 2009. Endocrine regulation of aging and reproduction in *Drosophila*. Special Issue: *The Endocrinology of Aging* **299**:39–50. DOI: https://doi.org/10.1016/j.mce.2008.07.005

Treherne JE, Schofield PK. 1981. Mechanisms of ionic homeostasis in the central nervous system of an insect. *Journal of Experimental Biology* **95**:61–73. DOI: https://doi.org/10.1242/jeb.95.1.61

Trettin J, Haubner M, Buschinger A, Heinze J. 2011. Queen dominance and worker policing control reproduction in a threatened ant. *BMC Ecology* **11**:21. DOI: https://doi.org/10.1186/1472-6785-11-21

Trible W, Chandra V, Lacy KD, Limón G, McKenzie SK, Olivos-Cisneros L, Arsenault SV, Kronauer DJC. 2023. A caste differentiation mutant elucidates the evolution of

socially parasitic ants. *Current Biology* **33**:1047-1058.e4. DOI: https://doi.org/10.1016/j.cub.2023.01.067

Tschinkel W. 1988. Colony growth and the ontogeny of worker polymorphism in the fire ant, *Solenopsis invicta*. *Behavioral Ecology and Sociobiology* **22**:103–115. DOI: https://doi.org/10.1007/BF00303545

Tsuji K, Nakata K, Heinze J. 1996. Lifespan and reproduction in a queenless ant. *Naturwissenschaften* **83**:577–578. DOI: https://doi.org/10.1007/BF01141985

Turner S. 2018. qqman: an R package for visualizing GWAS results using Q-Q and manhattan plots. *Journal of Open Source Software* **3**:731. DOI: https://doi.org/10.21105/joss.00731

Ulrich Y, Burns D, Libbrecht R, Kronauer DJC. 2016. Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. *Behavioral Ecology and Sociobiology* **70**:1011–1018. DOI: https://doi.org/10.1007/s00265-015-2046-2

Ulrich Y, Saragosti J, Tokita CK, Tarnita CE, Kronauer DJC. 2018. Fitness benefits and emergent division of labour at the onset of group living. *Nature* **560**:635–638. DOI: https://doi.org/10.1038/s41586-018-0422-6

Van Oystaeyen A, Oliveira RC, Holman L, van Zweden JS, Romero C, Oi CA, d'Ettorre P, Khalesi M, Billen J, Wäckers F, Millar JG, Wenseleers T. 2014. Conserved class of queen pheromones stops social insect workers from reproducing. *Science* **343**:287–290. DOI: https://doi.org/10.1126/science.1244899

Veale AJ, Foster BJ, Dearden PK, Waters JM. 2018. Genotyping-by-sequencing supports a genetic basis for wing reduction in an alpine New Zealand stonefly. *Scientific Reports* **8**:16275. DOI: https://doi.org/10.1038/s41598-018-34123-1

Viljakainen L, Jurvansuu J, Holmberg I, Pamminger T, Erler S, Cremer S. 2018. Social environment affects the transcriptomic response to bacteria in ant queens. *Ecology and Evolution* **8**:11031–11070. DOI: https://doi.org/10.1002/ece3.4573

Vizueta J, Xiong Z, Ding G, Larsen RS, Ran H, Gao Q, Stiller J, Dai W, Jiang W, Zhao J, Guo C, Zhang X, Zuo D, Zhong W, Schiøtt M, Liu C, Zhang H, Dai X, Andreu I, Shi Y, Tretter S, He D, Gautam S, Li Z, Hickey G, Ivens ABF, Meurville M-P, Hita-Garcia F, Kass JM, Guénard B, Moreau C, Paten B, LeBoeuf AC, Economo EP, Chapuisat M, Shik JZ, Ward PS, Heinze J, Schultz TR, Li Q, Dunn RR, Sanders NJ, Liu W, Schrader L,

Boomsma JJ, Zhang G. 2025. Adaptive radiation and social evolution of the ants. *Cell*. DOI: https://doi.org/10.1016/j.cell.2025.05.030

Von Wyschetzki K, Rueppell O, Oettler J, Heinze J. 2015. Transcriptomic signatures mirror the lack of the fecundity/longevity trade-off in ant queens. *Molecular biology and evolution*. DOI: https://doi.org/10.1093/molbev/msv186

von Zglinicki T, Bürkle A, Kirkwood TB. 2001. Stress, DNA damage and ageing—an integrative approach. *Experimental gerontology* **36**:1049–1062. DOI: 10.1016/s0531-5565(01)00111-5

von Zglinicki T, Saretzki G, Döcke W, Lotze C. 1995. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Experimental cell research* **220**:186–193. DOI: 10.1006/excr.1995.1305

Walton A, Herman JJ, Rueppell O. 2024. Social life results in social stress protection: a novel concept to explain individual life-history patterns in social insects. *Biological Reviews* **99**:1444–1457. DOI: https://doi.org/10.1111/brv.13074

Wang J, Wurm Y, Nipitwattanaphon M, Riba-Grognuz O, Huang Y-C, Shoemaker D, Keller L. 2013. A Y-like social chromosome causes alternative colony organization in fire ants. *Nature* **493**:664–668. DOI: https://doi.org/10.1038/nature11832

Wang X, Jiang Q, Song Y, He Z, Zhang H, Song M, Zhang X, Dai Y, Karalay O, Dieterich C, Antebi A, Wu L, Han JJ, Shen Y. 2022. Ageing induces tissue-specific transcriptomic changes in *Caenorhabditis elegans*. *The EMBO Journal* **41**:e109633. DOI: https://doi.org/10.15252/embj.2021109633

Warner MR, Mikheyev AS, Linksvayer TA. 2017. Genomic signature of kin selection in an ant with obligately sterile workers. *Molecular Biology and Evolution* **34**:1780–1787. DOI: https://doi.org/10.1093/molbev/msx123

Weger AA, Rittschof CC. 2024. The diverse roles of insulin signaling in insect behavior. F*rontiers in Insect Science* **Volume 4**-2024. DOI: 10.3389/finsc.2024.1360320

Weindruch R, Walford RL. 1988. The retardation of aging and disease by dietary restriction. Springfield, Ill., U.S.A: Thomas

Wenseleers T, Ratnieks FLW. 2006. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. T*he American Naturalist* 168:E163–E179. DOI: https://doi.org/10.1086/508619

References

West-Eberhard MJ. 2003. Developmental plasticity and evolution. *Oxford University* Press.

Westendorp RGJ, Kirkwood TBL. 1998. Human longevity at the cost of reproductive success. *Nature* 396:743–746. DOI: https://doi.org/10.1038/25519

Wheeler WM. 1911. The ant-colony as an organism. *Journal of Morphology* 22:307–325. DOI: https://doi.org/10.1002/jmor.1050220206

Wickham H. 2016. Data analysis. Ggplot2: Elegant graphics for data analysis. *Springer*. p. 189–201. See: https://ggplot2.tidyverse.org

Williams GC. 1957. Pleiotropy, natural selection, and the evolution of senescence. *Evolution* 11:398–411. DOI: https://doi.org/10.2307/2406060

Wilson EO. 1985. The sociogenesis of insect colonies. *Science* 228:1489–1495. DOI: https://doi.org/10.1126/science.228.4707.1489

Wilson EO. 1980. Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *Behavioral Ecology and Sociobiology* 7:157–165. DOI: https://doi.org/10.1007/BF00299521

Wilson EO. 1971. The insect societies. *Harvard PaperbacksBelknap* Press of Harvard University Press.

Wilson EO, Hölldobler B. 2005. Eusociality: origin and consequences. *Proceedings of the National Academy of Sciences* 102:13367–13371. DOI: https://doi.org/10.1073/pnas.0505858102

Woodcox A. 2018. Aristotle's theory of aging. *Cahiers des études anciennes*, LV 65–78. See: https://journals.openedition.org/etudesanciennes/1040

Woyke J. 1971. Correlations between the age at which honeybee brood was grafted, characteristics of the resultant queens, and results of insemination. *Journal of Apicultural Research* **10**:45–55. DOI: https://doi.org/10.1080/00218839.1971.11099669

Wu Z, Libin Y, He Q, Zhou S-T. 2021. Regulatory mechanisms of vitellogenesis in insects. *Frontiers in Cell and Developmental Biology* **8**. DOI: https://doi.org/10.3389/fcell.2020.593613

Wyatt CDR, Bentley MA, Taylor D, Favreau E, Brock RE, Taylor BA, Bell E, Leadbeater E, Sumner S. 2023. Social complexity, life-history and lineage influence the

molecular basis of castes in vespid wasps. *Nature Communications* **14**:1046. DOI: https://doi.org/10.1038/s41467-023-36456-6

Wyatt GR, Davey KG. 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. In: Evans PD (Ed). *Advances in Insect Physiology*. Academic Press. p. 1–155. DOI: https://doi.org/10.1016/S0065-2806(08)60030-2

Yagound B, Blacher P, Fresneau D, Poteaux C, Châline N. 2014. Status discrimination through fertility signalling allows ants to regulate reproductive conflicts. *Animal Behaviour* 93:25–35.

Yamamoto R, Bai H, Dolezal AG, Amdam G, Tatar M. 2013. Juvenile hormone regulation of *Drosophila* aging. *BMC Biology* **11**:85. DOI: https://doi.org/10.1186/1741-7007-11-85

Yamamoto R, Chung R, Vazquez JM, Sheng H, Steinberg PL, Ioannidis NM, Sudmant PH. 2022. Tissue-specific impacts of aging and genetics on gene expression patterns in humans. *Nature Communications* **13**:5803. DOI: https://doi.org/10.1038/s41467-022-33509-0

Yan H, Opachaloemphan C, Carmona-Aldana F, Mancini G, Mlejnek J, Descostes N, Sieriebriennikov B, Leibholz A, Zhou X, Ding L, Traficante M, Desplan C, Reinberg D. 2022. Insulin signaling in the long-lived reproductive caste of ants. *Science* **377**:1092–1099. DOI: https://doi.org/10.1126/science.abm8767

Yang C-H, Andrew Pospisilik J. 2019. Polyphenism – A window into gene-environment interactions and phenotypic plasticity. *Frontiers in Genetics* Volume **10**-2019.

Yi J, Li F, Xu C, Liu Y, Hou M. 2021. Expression analyses of vitellogenin and target of rapamycin of *Sogatella furcifera* (Hemiptera: Delphacidae), and their effects on reproduction. J*ournal of Economic Entomology* **114**:2562–2570. DOI: https://doi.org/10.1093/jee/toab195

Zdbicka-Barabas A, Cytrynska M. 2013. Apolipophorins and insects immune response. I*nvertebr. Surviv. J.* **10**:58–68.

Zeng H. 2023. Functional properties of ant queen pheromones as revealed by behavioral experiments. *Behavioral Ecology and Sociobiology* **77**:113. DOI: https://doi.org/10.1007/s00265-023-03378-8

References

Zhao Y, Liu W, Zhao X, Yu Z, Guo H, Yang Y, Zhang Jianqin, Moussian B, Zhang Jianzhen. 2020. Apolipophorin-II/I contributes to cuticular hydrocarbon transport and cuticle barrier construction in *Locusta migratoria*. *Front Physiol*. DOI: 10.3389/fphys.2020.00790.

Zheng X, Levine D, Shen J, Gogarten SM, Laurie C, Weir BS. 2012. A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics* **28**:3326–3328. DOI: https://doi.org/10.1093/bioinformatics/bts606

Zhou X, Dou Q, Fan G, Zhang Q, Sanderford M, Kaya A, Johnson J, Karlsson EK, Tian X, Mikhalchenko A, Kumar S, Seluanov A, Zhang ZD, Gorbunova V, Liu X, Gladyshev VN. 2020. Beaver and naked mole rat genomes reveal common paths to longevity. *Cell Reports* **32**. DOI: https://doi.org/10.1016/j.celrep.2020.107949

Zhu P, Liu W, Zhang X, Li Meng, Liu G, Yu Y, Li Z, Li X, Du J, Wang X, Grueter CC, Li Ming, Zhou X. 2023. Correlated evolution of social organization and lifespan in mammals. *Nature Communications* **14**:372. DOI: https://doi.org/10.1038/s41467-023-35869-7

Zhu S, Liu F, Zeng H, Li N, Ren C, Su Y, Zhou S, Wang G, Palli SR, Wang J, Qin Y, Li S. 2020. Insulin/IGF signaling and TORC1 promote vitellogenesis via inducing juvenile hormone biosynthesis in the American cockroach. *Development* **147**:dev188805. DOI: https://doi.org/10.1242/dev.188805

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